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Gremlin un nuevo mediador en la progresión de la enfermedad renal.

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Gremlin un nuevo mediador en la progresión de la enfermedad renal.

Memoria que presenta la licenciada en Bioquímica
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A mi papá

“En tiempos de crisis la imaginación es más efectiva que el intelecto”

Albert Einstein

“Haz de tu vida un sueño, y de tu sueño una realidad”

Antoine de Saint-Exupéry

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RESUMEN

Gremlin es un miembro de la superfamilia de TGF-β que actúa como antagonista de las proteínas morfogenéticas de hueso (BMPs), participando en desarrollo embrionario y fibrosis. Recientemente se han demostrado efectos celulares directos de Gremlin independientes de BMPs, algunos de ellos mediados por el receptor VEGFR2 (*Vascular Endotelial growth factor receptor-2*). Gremlin se re-expresa en adultos en condiciones patológicas, y como ha descrito nuestro grupo, en enfermedades renales crónicas asociado a fibrosis y pérdida de funcionalidad. El objetivo de esta tesis ha sido determinar los efectos de Gremlin en riñón en condiciones fisiológicas y patológicas, investigando el receptor implicado y los mecanismos intracelulares activados, con especial atención a la vía Notch. Los resultados demuestran que Gremlin se une y activa el receptor VEGFR2 y modula la respuesta inflamatoria renal por activación temprana de la vía canónica de NF-κB. Esta activación induce la expresión de factores pro-inflamatorios e infiltración renal de monocitos/macrófagos y linfocitos. *In vitro* hemos observado que Gremlin, vía VEGFR2 regula factores pro-fibróticos, componentes de matriz extracelular y eventos asociados a transición epitelio-mesenquimal. Además, Gremlin es un mediador de las acciones pro-fibróticas de TGF-β. En modelos experimentales de daño renal y en patologías renales crónicas humanas se induce Gremlin y se activa la vía del VEGFR2 en riñón, sugiriendo que el eje Gremlin/VEGFR2 podría contribuir a la progresión del daño renal. Así, el bloqueo de VEGFR2 retardó la progresión del daño renal experimental, inhibiendo Gremlin y disminuyendo inflamación y fibrosis, mientras que el ligando canónico de VEGFR2 no fue modificado. La regulación de estos procesos es compleja e involucra la activación y la integración de otros sistemas de señalización intracelular. Gremlin activa la vía de señalización Notch asociado a la regulación de factores pro-fibróticos y marcadores de transición epitelio mesenquimal. El bloqueo de la ruta Notch mejoró el daño renal experimental inhibiendo la fibrosis. En pacientes con nefropatías progresivas se observó activación de la vía Notch, pero no en nefropatía hipertensiva o en modelos experimentales de hipertensión. En estudios *in vivo e in vitro,* Angiotensina II no activó la ruta Notch, mostrando una clara diferencia con otros factores pro-fibróticos, como TGF-β y Gremlin. En conjunto, estos datos sugieren que Gremlin podría ser considerado un nuevo mediador de daño renal a través de la activación de VEGFR2, y sugieren el eje Gremlin/VEGFR2 como una nueva diana terapéutica para enfermedades renales crónicas.

SUMMARY

Gremlin is a member of the TGF-β superfamily and a bone morphogenetic proteins (BMPs) antagonist involved in embryonic development and fibrosis. BMPs independent Gremlin cellular effects have recently been described, some of them mediated by binding to VEGFR2 receptor (*vascular endothelial growth factor receptor*-2). Gremlin is re-expressed in pathological conditions in adults, as previously described by our group in chronic kidney diseases associated with fibrosis and renal failure. The aim of this thesis was to determine the Gremlin actions in the kidney, investigating the receptor involved and the intracellular mechanisms activated, with special attention to the Notch pathway. The results demonstrate that Gremlin binds to and activates the VEGFR2 receptor signaling linked to renal inflammation. Gremlin caused an early activation of the canonical NF-kB pathway and upregulation of pro-inflammatory factors, leading to infiltration of monocytes/macrophages and lymphocytes into the kidney. *In vitro* Gremlin, via VEGFR2, regulated pro-fibrotic factors, extracellular matrix components and epithelial-mesenchymal transition related-events. Moreover, Gremlin is a downstream mediator of TGF-β pro-fibrotic actions. In experimental models of renal injury and in human chronic kidney pathologies, Gremlin induction and VEGFR2 pathway activation in the kidney was found, suggesting that Gremlin/VEGFR2 axis could contribute to the progression of renal damage. Thus, VEGFR2 blockade delayed renal damage progression, associated to Gremlin downregulation, while the canonical VEGFR2 ligand was not modified. The regulation of this process is complex and involves the activation and integration of other intracellular signaling systems. Gremlin activated the Notch signaling pathway in the kidney associated to the up-regulation of pro-fibrotic factors and changes in epithelial-mesenchymal transition markers. Notch pathway blockade improved experimental renal damage, diminishing fibrosis. In patients with several progressive nephropathies, except in hypertensive nephropathy, renal Notch pathway activation was found. In response to Angiotensin II *in vivo* and *in vitro* Notch pathway was not activated, showing a clear difference with others pro-fibrotic factors, such as TGF-β and Gremlin. In sumary, these data suggest that Gremlin through VEGFR2 activation could be considered a novel mediator of kidney damage, and suggests that the Gremlin/VEGFR2 axis could be as a novel therapeutic target for chronic kidney diseases.

ÍNDICE

Abreviaturas

En algunos casos se ha conservado la correspondiente abreviatura en inglés debido a su frecuente utilización en el lenguaje científico.

α-SMA	α-actina de músculo liso
AP-1	Proteína activada-1
Ang II	Angiotensina II
ARAII	Antagonistas de los receptores de la Ang II
BMP	Proteína morfogenética del hueso
CXCL	*Chemokine (C-X-C motif) ligand*
CTGF	Factor de crecimiento de tejido conectivo
DHR	dominio de homología Rel
Drm	*Down regulated by mos*
ECA	Enzima convertidora de angiotensina
EGF	Factor de crecimiento endotelial
ERC	Enfermedad renal crónica
ET-1	Endotelina-1
FRA	Fracaso renal agudo
FGF-2	Factor de crecimiento de fibroblastos-2
FLK-1	*Fetal liver kinase-1*
FSP-1	Proteína específica de fibroblasto-1
FTI	Fibrosis túbulo-intersticial
ICAM-1	Molécula de adhesión intercelular-1
IGF	Factor de crecimiento tipo insulina
IHG-2	*induced in high glucose-2*
IL-1β	Interleuquina-1β
IL-6	Interleuquina-6
HES	*Hairy/Enhancer of split*
HERP	*HES-related repressor protein*
HIF-1	Factor inducible por hipoxia-1
HK2	Células túbulo-epiteliales proximales humanas inmortalizadas
IECAs	Inhibidores de la Enzima convertidora de Ang II
KDR	*kinase insert domain containing receptor*
LAP	Péptido asociado a latencia
LTBPs	Proteína de unión a TGF-β latente
MCP	Proteína quimioatractante de monocitos
MAPK	Proteínas quinasas activadas por mitógeno
MEC	Matriz extracelular
MIF	Factor inhibitorio de la migración de macrófagos
MIP	Proteína inflamatoria de macrófagos
MMPs	Metaloproteinasas
NF-κB	Factor nuclear-κB
ND	Nefropatía diabética
NH	Nefropatía hipertensiva
NICD	Dominio intracelular de Notch
PDGF	Factor de crecimiento derivado de plaquetas
Rantes	*Regulated on Activation, Normal T Expressed and Secreted*

RBP-Jκ	*Recombination Signal-Binding Protein 1 for J-Kappa*
R-Smads	Smads reguladas por el receptor
ROCK	Proteína quinasa asociada a Rho
ROS	Especies reactivas de oxígeno
SBE	Elemento de unión a Smad
SLN	Secuencia de localización nuclear
SRAA	Sistema renina angiotensina aldosterona
SHR	Ratas espontáneamente hipertensas
STZ	Estreptozotocina
TEM	Transición epitelio-mesenquimal
TEnM	Transición endotelio-mesenquimal
TFBs	Fibroblastos de ratón
TME	Transición mesénquimo-epitelial
TGF-β	Factor de crecimiento transformante-β
TNF-α	Factor de necrosis tumoral-α
TRI	Receptor de TGF-β tipo I
TRII	Receptor de TGF-β tipo II
Tweak	*TNF-like weak inducer of apoptosis*
UUO	Obstrucción ureteral unilateral
VCAM	V*ascular cell adhesion molecule-1*
VEGFA	Vascular e*ndotelial growth factor-A*
VEGFR2	Vascular e*ndotelial growth factor receptor-2*

I. INTRODUCCIÓN

1. Enfermedad renal crónica

La enfermedad renal crónica (ERC) es un importante problema de salud pública de la sociedad moderna tanto por su elevada incidencia y prevalencia como por su alto coste socio-económico.[60,90] Esta patología no sólo involucra daño renal sino también un elevado riesgo de morbi-mortalidad, sobre todo de naturaleza cardiovascular.[39,40]

Existen varios factores involucrados en el aumento de pacientes con ERC, los que incluyen la creciente incidencia de diabetes mellitus tipo 2 (y consecuentemente la nefropatía diabética), la hipertensión arterial, la obesidad y el envejecimiento de la población.[16,37,70,168] Tratamientos clínicos disponibles para la ERC sólo retardan la progresión de la enfermedad. Además no hay biomarcadores que reflejen la gravedad de los cambios histopatológicos renales subyacentes y que puedan predecir eficazmente la progresión de la ERC hacia la etapa terminal de la enfermedad renal.[131,170] Uno de los mayores problemas actuales es el número creciente de pacientes que progresan hasta insuficiencia renal terminal y requieren tratamiento de sustitución mediante diálisis (hemodiálisis o diálisis peritoneal) y posteriormente trasplante, lo que conduce a un deterioro en la calidad de vida de estos pacientes y un alto coste para los sistemas de salud.[3,50]

La fibrosis es uno de los grandes grupos de enfermedades para las cuales no existe terapia, cabe destacar que casi el 45% de todas las muertes que ocurren en el mundo desarrollado son causadas por desórdenes inflamatorios y fibrogénicos crónicos, que incluyen la fibrosis pulmonar, renal y hepática.[180] Por otra parte, el fracaso renal agudo (FRA), si es mantenido, puede conducir a la fibrosis renal.[83,258]

La mayoría de las nefropatías progresan lentamente hacia la pérdida definitiva de la función renal. Esta progresión se caracteriza por un proceso inflamatorio inicial, seguido de fibrosis túbulo-intersticial (FTI), atrofia tubular y glomérulo-esclerosis.[187] Las células renales residentes desempeñan un papel importante en el proceso inflamatorio y fibrótico que tiene lugar en estas patologías renales. Participan en las etapas tempranas del daño renal ya que son capaces de producir mediadores inflamatorios, moléculas de adhesión y factores de crecimiento; y además pueden contribuir a la progresión del daño crónico, promoviendo la síntesis y acumulación de matriz extracelular (MEC) que dan lugar al estado fibrótico.[87,121,199,200,277] (**Figura 1**) Entre los factores más importantes de daño túbulo-intersticial

destacan la proteinuria y la activación del sistema renina angiotensina aldosterona (SRAA).[146] Este sistema tiene un papel fundamental en los cambios fisiopatológicos que llevan al fallo renal terminal. Las investigaciones realizadas en los últimos años han mostrado su complejidad y su principal efector, la Angiotensina II (Ang II) ha pasado de ser considerada como un agente vasoactivo a una verdadera citoquina pro-inflamatoria y pro-fibrótica.[200,254]

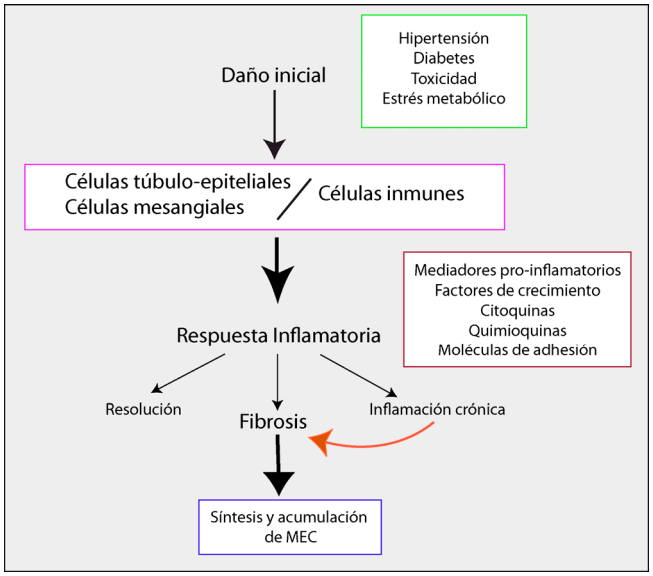

Figura 1: *Esquema de los mecanismos celulares y moleculares que se producen en respuesta a un daño.*

Los tratamientos clínicos empleados en la actualidad sólo consiguen frenar la progresión del daño renal, pero no revierten las lesiones. Todo esto hace evidente la importante necesidad de estudiar los mecanismos moleculares y celulares que conllevan a la insuficiencia renal crónica y sus posibles dianas y tratamientos para minimizar su progresión y tratar las complicaciones derivadas.

2. Proceso inflamatorio renal

Después de una lesión inicial, el riñón afectado sufre una serie de eventos en un intento por reparar y/o recuperarse del daño. En el riñón, el proceso inflamatorio se caracteriza por infiltración glomerular y túbulo-intersticial de células del sistema inmune, incluyendo neutrófilos, macrófagos, linfocitos y mastocitos.[14,55,121]

En condiciones patológicas, las células renales se activan y comienzan a liberar citoquinas (IL-6, IL-1β, TNF-α, Tweak), péptidos vasoactivos (Ang II, ET-1) y factores de crecimiento (TGF-β, CTGF, PDGF) que intervienen en la producción de quimioquinas (MCP-1,2-3, Rantes, CXCL-16, CXCL-1, MIP-1,2-3) y moléculas de adhesión (ICAM-1). La activación de estas respuestas se produce a través de diferentes vías de señalización entre las que destacan la vía del factor nuclear-κB (NF-κB), la vía de la ruta de las quinasas activadas por mitógeno (MAPKs) y las especies reactivas de oxígeno (ROS).[18,19,34,66,142,189,197,212,218,251] El aumento en la expresión de estas moléculas favorece la infiltración de células inflamatorias (macrófagos y linfocitos) y la diferenciación y proliferación de miofibroblastos, lo que contribuye a la progresión de la inflamación y, en último término, a la fibrosis.[112,218] La inflamación persistente es la causa subyacente que contribuye activamente a la enfermedad renal crónica.[123,277]

2.1. Vía del Factor Nuclear-κB (NF-κB)

NF-κB es una familia de factores de transcripción pleiotrópicos, que tiene un rol esencial en inflamación, apoptosis, inmunidad, proliferación celular y diferenciación.[9,71,212] La vía del NF-κB se encuentra presente en todos los tipos celulares, y en el riñón se ha descrito su activación en podocitos, células mesangiales, tubulares y endoteliales.[212] Es un factor de trascripción de respuesta rápida, que responde ante una gran variedad de estímulos como por ejemplo, productos virales o bacterianos, factores de crecimiento, citoquinas inflamatorias como TNF-α, IL-1β, el péptido pleiotrópico Ang II, estrés intracelular como sobrecarga proteica en el retículo endoplásmico, estrés extracelular como la luz ultravioleta y estrés metabólico como altas concentraciones de glucosa y productos de glicosilación avanzada.[22,66,71,212]

En mamíferos se han identificado cinco miembros de NF-κB: p50, p65 (RelA), c-Rel, p52 y RelB que se encuentran en forma de homodímeros o heterodímeros.[71] Todas estas proteínas presentan en el extremo N-terminal una región muy conservada de aproximadamente 300 aminoácidos denominada dominio de homología Rel (DHR), a través de la cual se une al ADN, dimeriza e interacciona con los miembros de la familia de proteínas inhibitorias IκB. Además DHR contiene una señal de localización nuclear (SLN) que facilita la translocación del NF-κB al núcleo.[246]

En células en reposo, los dímeros de NF-kB se encuentran en el citoplasma unidos a una subunidad inhibidora llamada IκB. Esta familia está compuesta por las proteínas IκBα, IκBβ,

IκBγ, IκBδ, IκBε y Bcl-3. Las IκB contienen entre cinco y siete repeticiones conservadas de tipo anquirina, que son fundamentales para su unión a NF-κB a través de los dominios DHR. De este modo, el represor o la subunidad inhibitoria enmascaran la SLN e impiden su translocación al núcleo. Las proteínas IκB, poseen además una secuencia de desestabilización en la región C-terminal, que favorece su degradación proteolítica.[61,71]

El dímero más abundante en la mayoría de los tipos celulares y por lo tanto el más estudiado, es el formado por las subunidades p65/p50, que está predominantemente unido a la proteína inhibitoria IκBα. La activación de la vía canónica del NF-κB involucra la activación de las quinasas IKKs (IKKα, IKKβ y la subunidad reguladora NEMO), que inducen la fosforilación de la subunidad inhibitoria IκB (principalmente de IκBα) en los residuos de Ser32 y Ser36 que marcan a esta proteína para ser reconocida por el complejo ligasa-ubiquitina.[68,178] Esta enzima poliubiquitina a IκB en la Lys21 y Lys22, lo que provoca su degradación por el proteasoma 26S. Este proceso permite el desenmascaramiento de la SLN de NF-κB, la fosforilación de la subunidad NF-κB p65 en la Ser536 por IKKs,[38,71] lo que resulta en su translocación al núcleo, activando la transcripción de genes diana debido a la unión a los elementos de alta afinidad -κB en sus promotores y la formación de complejos con varios coactivadores.[71,178,246] (**Figura 2**) Entre los genes que regula NF-κB por la vía canónica se encuentran genes implicados en la respuesta inmune e inflamatoria como VCAM-1, MCP-1, Rantes, TNF-α e IL-6, en el remodelado tisular como las metaloproteinasas de matriz (MMPs), y enzimas como la fosfolipasa 2A y ciclooxigenasa.[47,94,135,179,195,212,261]

Por su parte, la vía no canónica de NF-κB se caracteriza por el procesamiento de NF-κB2/p100, que depende de la activación de la quinasa IKK-α por la quinasa inductora de NF-κB[183] dando lugar al complejo de p52/RelB, que se trasloca directamente al núcleo.[45] Entre los genes diana de NF-κB2, se encuentran el factor quimiotáctico de linfocitos T, CCL21.[207] Además existe una vía híbrida que requiere la contribución de ambas vías, en donde la vía no canónica genera el complejo y la vía canónica activa el complejo.[45]

En enfermedades renales humanas como nefropatía diabética, nefropatía por IgA, glomerulonefritis crescéntica, nefritis lúpica entre otras, existe evidencia histológica de la activación de la vía clásica de NF-κB.[6,66,145,206,284] En modelos experimentales de glomerulonefritis inmune y de obstrucción unilateral del uréter (UUO), la inhibición de esta vía

de señalización mediante el bloqueo farmacológico con Parthenolide (inhibidor de la fosforilación de IκB), produce una mejora en la inflamación renal.[53,124] Parthenolide también disminuye la acumulación de monocitos intersticiales en el daño renal experimental mediado por Ang II y por cisplatino y en ateroesclerosis.[53,58,125]

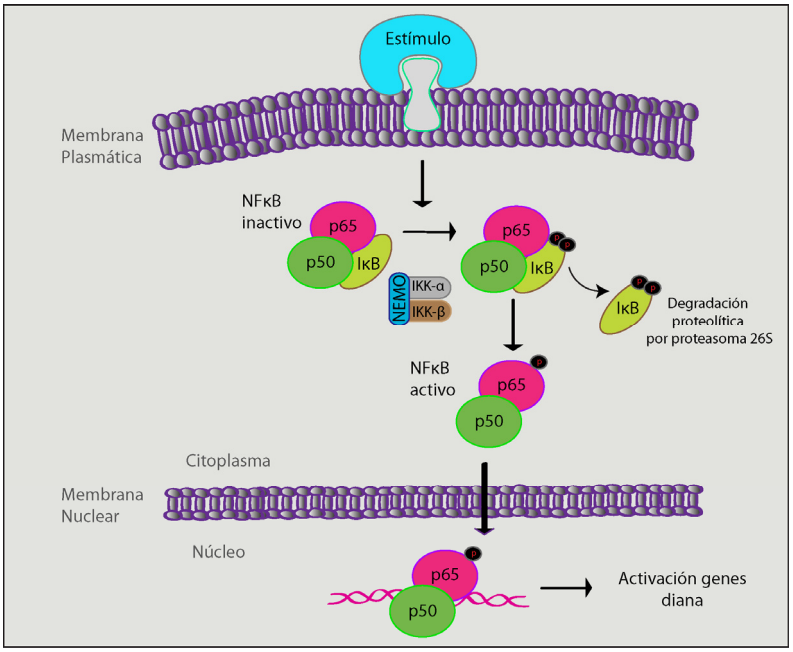

Figura 2*: Vía canónica de la activación de NF-κB.*

3. Fibrosis Renal

La respuesta normal del riñón tras un daño, es la reparación con el fin de lograr la restauración completa de la función y arquitectura del tejido. La reparación sin cicatriz ocurre únicamente en tejido fetal, en adulto sólo se logran grados variables de restauración del tejido. Es por esto que a pesar de la reparación fisiológica, la fibrogénesis renal no termina, y carece de una verdadera fase de resolución. De hecho en el tejido adulto el proceso persiste independiente del daño inflamatorio inicial, ocasionando un depósito excesivo de MEC y así consecuentemente la destrucción de la morfología renal y la alteración de la función del órgano.[74,139,283]

Los hallazgos patológicos de la fibrosis renal son descritos como glomérulo-esclerosis, FTI, infiltración inflamatoria y nefro-esclerosis, estos eventos conducen a una pérdida del parénquima renal caracterizado por atrofia tubular y disminución del número de capilares. Los

eventos celulares fundamentales que llevan a estos hallazgos histológicos son aún más complejos e incluyen: activación de células mesangiales y fibroblastos, apoptosis, transición epitelio-mesenquimal (TEM), infiltración de monocitos, macrófagos y linfocitos T.[121,122]

El proceso de fibrogénesis renal es mediado principalmente por células túbulo-epiteliales, el tipo celular predominante del túbulo-intersticio, por células mononucleares pro-inflamatorias y por fibroblastos intersticiales, estas últimas son las células profesionales que sintetizan MEC. Este proceso se divide en:

• Fase inflamatoria, que se inicia con la activación de las células epiteliales tubulares o de las células mononucleares infiltradas.

• Incremento en el número de miofibroblastos intersticiales, lo que lleva a un aumento en el depósito de componentes de la MEC siendo un proceso reversible y similar a la cicatrización de una herida.[267] Cuando su producción es excesiva, es deletérea ya que exacerba el daño creando un círculo vicioso de perpetuación.[74,277]

La FTI se vincula a un desequilibrio patológico entre la síntesis y la degradación de la MEC en respuesta a una inflamación persistente y se estimula por una variedad de citoquinas y factores de crecimiento.[255] En la FTI se produce una acumulación excesiva de los componentes de la MEC que incluyen Colágeno tipo I y III, así como proteoglicanos y Fibronectina producidos por fibroblastos activados.[276] En este contexto, los miofibroblastos son el tipo celular que representa el fenotipo de fibroblasto activado durante el daño, expresan α-actina de músculo liso (α-SMA)[185,227,228] y el aumento en su número se correlaciona con la progresión de la enfermedad renal.[98,140,183] Estudios *in vitro* enfatizan la importancia de la activación de los fibroblastos como un evento celular particular, pero este tipo celular aislado no es capaz de iniciar y mantener en total escala la fibrosis renal, de manera que es necesaria la participación e interacción de otros tipos de células infiltradas y residentes del riñón.[121]

La FTI ha sido considerada un denominador común de progresión de la enfermedad renal crónica independientemente de su etiología. Este proceso juega un rol clave en la progresión del daño y es así como la severidad de los cambios túbulo-intersticiales se correlacionan mejor con la pérdida de función renal que con el grado de glomérulo-esclerosis, lo que no es sorprendente ya que el túbulo-intersticio ocupa más del 90% del volumen renal.[74,276]

El origen de los fibroblastos intersticiales ha sido tema de discusión **(Figura 3)**, siendo muchos los orígenes que se han propuesto.[111] Algunos autores han postulado que estos derivarían de leucocitos migratorios, mientras que otros proponen que los fibroblastos procederían de células intersticiales residentes en el riñón.[227,228] Otra hipótesis argumenta que son las células de la médula ósea las progenitoras de los fibroblastos intersticiales, que a través de la circulación sistémica alcanzan y pueblan los órganos periféricos.[80]

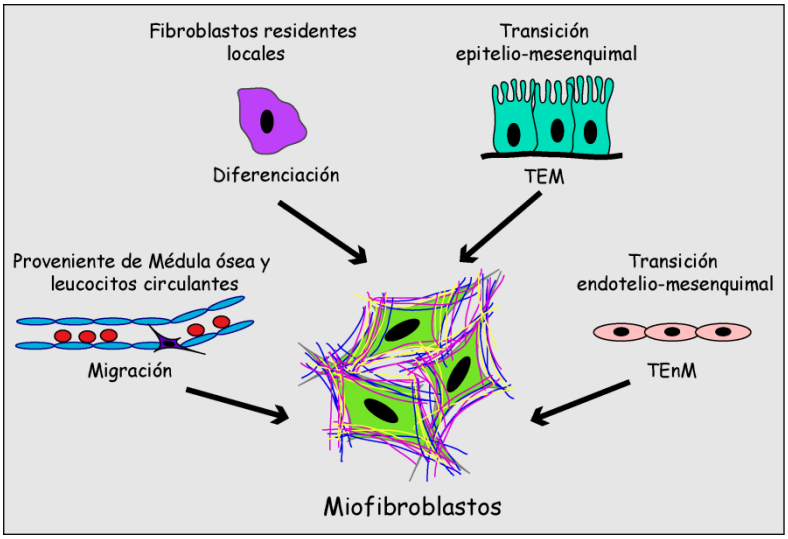

Figura 3: *Origen de los miofibroblastos.*

Estudios en cultivos celulares y nefropatías experimentales han planteado que a partir del proceso de TEM[120,185,272] o transición endotelio mesenquimal (TEnM)[273] células epiteliales o endoteliales se pueden convertir en verdaderos fibroblastos, produciendo proteínas de la MEC que son capaces de migrar al intersticio comportándose como células productoras de Colágeno.[87,164,185]

3.1. Transición epitelio-mesenquimal

Clásicamente se ha considerado que la expresión génica es un fenómeno irreversible establecido en el momento de la replicación celular. Sin embargo, se ha planteado que las células diferenciadas están dotadas con la capacidad de transformarse en células de diferente tipo adquiriendo otras funciones, caracterizándose la transición celular por pérdida y adquisición de nuevos fenotipos.[8,93,234,274] El cambio celular entre fenotipo epitelial y mesenquimal, por *turn on/off* de genes específicos durante el desarrollo temprano, es un

proceso reconocido que caracteriza la plasticidad embrionaria.[115,234] Es así, como a partir del epiblasto y mediante sucesivos procesos de TEM y de transición mesénquimal-epitelial (TME), se dará origen a células de tejido conectivo y epitelial.[91,185] De esta forma, el epitelio tubular, los conductos y los fibroblastos renales son generados vía transiciones celulares durante el desarrollo y crecimiento normal del órgano.[164]

La transformación neoplásica del fenotipo epitelial al mesenquimal con capacidad invasiva y migratoria a través de la MEC, pone en evidencia el importante rol que presenta la TEM en condiciones patológicas como el cáncer.[264,265,266] Por último, evidencias de transición han sido observadas en la mayoría de los tejidos maduros, donde el proceso parece estar principalmente relacionado a la cicatrización de heridas y remodelamiento fibrótico después de un proceso inflamatorio.[92,264,274]

A nivel renal, la TEM es un proceso en el cual las células epiteliales tubulares renales pierden su fenotipo epitelial y adquieren características de mesénquima, conversión fenotípica que fundamentalmente se asocia a la patogénesis de la fibrosis intersticial.[120] Uno de los primeros estudios en demostrar la presencia de TEM en riñón se realizó mediante análisis de comparación de tránscritos, en donde se aislaron varios genes específicos de fibroblastos, y encontraron que la proteína específica de fibroblasto-1 (Fsp-1) era un gen altamente específico de fibroblasto, que no es expresado en células epiteliales y está asociado con la conversión de células epiteliales a fenotipo fibroblástico.[226] Posteriormente, en un estudio con quimeras de médula ósea y ratones transgénicos, se demostró que un pequeño número de fibroblastos Fsp-1^+ migran al espacio intersticial normal desde la médula ósea y que una gran cantidad de fibroblastos Fsp-1^+ derivan de TEM local durante la fibrogénesis renal.[85]

Después del daño, es común encontrar en el riñón epitelio tubular que ha sufrido TEM, y aproximadamente entre el 25-50% de los fibroblastos renales son producidos localmente por TEM en respuesta a inflamación persistente.[85,111,164] Se han definido claramente cuatro eventos claves involucrados en la TEM: I) pérdida de la adhesión de las células epiteliales con disminución de la expresión de E-cadherina; II) expresión *de novo* de α-actina de músculo liso (α-SMA) y reorganización del citoesqueleto de actina, III) degradación de la membrana basal tubular y IV) aumento en la capacidad de invasión y migración celular.[256] **(Figura 4)**

Cabe destacar que la activación miofibroblástica es una respuesta fibrogénica temprana después de un proceso inflamatorio, mientras que la TEM a menudo se presenta como una forma más tardía.[121]

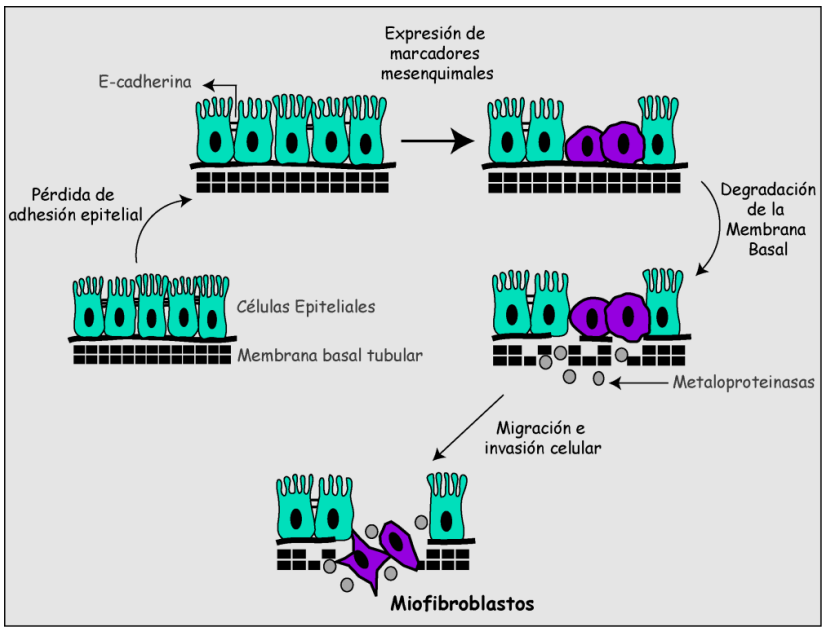

Figura 4: *Etapas de la transición epitelio-mesenquimal.*

Los moduladores de la TEM durante la inflamación renal involucran una combinación de citoquinas con actividad constante, entre las que se encuentran las proteínas Wnt, quinasas tipo integrinas, IGF I y II, EGF, FGF-2, TGF-β, Ang II, CTGF, y la participación de MMP-2 y MMP-9, también conocidas como inhibidores del ensamblaje de la membrana basal, que inician el proceso de degradación de la membrana a nivel local.[25,28,91,164,278] Entre los mecanismos de señalización claves en la TEM, destacan la activación de proteínas quinasas, como la cascada MAPKs (proteínas quinasas activadas por mitógeno) y la activación de la proteína G pequeña Rho y de su diana efectora ROCK (proteína quinasa asociada a Rho), y la activación del sistema de señalización de las proteínas Smad.[7,12,28,51,174,188]

3.2. Factor de Crecimiento Transformante-β

TGF-β pertenece a una superfamilia de factores de crecimiento formada por más de 40 miembros, entre los que se encuentran activinas, inhibinas, factores de crecimiento y de diferenciación, proteínas morfogenéticas del hueso (BMPs) y el factor de crecimiento

Mülleriano.[211,249] Se han descrito tres isoformas diferentes de TGF-β: TGF-β1, TGF-β2 y TGF-β3, siendo TGF-β1 la isoforma predominante involucrada en fibrosis renal.[262] Todos los miembros comparten una estructura dimérica y la presencia del motivo estructural nudo de cisteína. Además son reguladores multifuncionales de la división, migración, adhesión, organización y muerte celular, promueven la producción de MEC, la homeostasis y la embriogénesis.[89,129,130,155]

La síntesis de TGF-β1 es un proceso complejo. Su expresión génica se regula por Ang II, estrés mecánico y altas concentraciones de glucosa, todos ellos implicados en la progresión del daño renal.[201] TGF-β1 se sintetiza como una proteína inactiva llamada TGF-β latente, que consiste de una región principal y de un péptido asociado a latencia (LAP). Se encuentra en forma inactiva asociado a la MEC a través de proteínas de unión a TGF-β latente (LTBPs) que impiden la unión de TGF-β a su receptor.[239] La activación de TGF-β1, al liberarse el péptido asociado a latencia por activación de proteasas, puede estar mediada por Trombospondina-1, plasmina, pH ácido, ROS, MMPs o integrinas.[82,157]

TGF-β1 regula una gran cantidad de procesos biológicos tales como morfogénesis, desarrollo embrionario, cicatrización de heridas e inflamación. Las alteraciones, tanto en TGF-β1 como en los componentes de su vía de señalización, pueden contribuir a una amplia gama de enfermedades incluidas patologías renales, cardiovasculares, fibrosis, cáncer y enfermedades congénitas.[104,129,186,201,211]

TGF-β1 es considerado la principal citoquina pro-fibrogénica del riñón[20,277] y es capaz de estimular directamente la transcripción de un gran número de genes de MEC, inhibir la producción de colagenasas y estimular la expresión tisular del inhibidor de las metaloproteinasas, favoreciendo la acumulación de MEC.[130,163] Es conocido que TGF-β1 puede iniciar y mantener la TEM tanto *in vitro* como *in vivo*, a través de la activación de vías de señalización y reguladores transcripcionales que se encuentran integrados en extensas redes de señalización.[130,163,264]

En la vía canónica de activación de TGF-β1, la unión del ligando TGF-β activo al receptor de TGF-β tipo II (TRII), induce su actividad serin-treonina quinasa que fosforila al receptor de TGF-β tipo I (TRI), formándose un complejo ligando-receptor activado que propaga la señal al interior de la célula a través de la fosforilación de Smads reguladas por el receptor, como son Smad-2 y/o Smad-3 (R-Smads); que posteriormente forman heterocomplejos con la subunidad

reguladora Smad-4.[128,233] Los complejos Smad activados se translocan al núcleo y se unen a elementos reguladores conocidos como elementos de unión a SMAD (SBE), donde controlan la activación transcripcional de genes diana, actuando en conjunto con otros factores de transcripción coactivadores y correpresores.[128,239] El resultado de este complejo proceso es la proliferación de fibroblastos, sobre-expresión de Vimentina, α-SMA, MMP-2, MMP-9, Colágeno tipo I y tipo III y Fsp-1 entre otros genes.[164] (**Figura 5**)

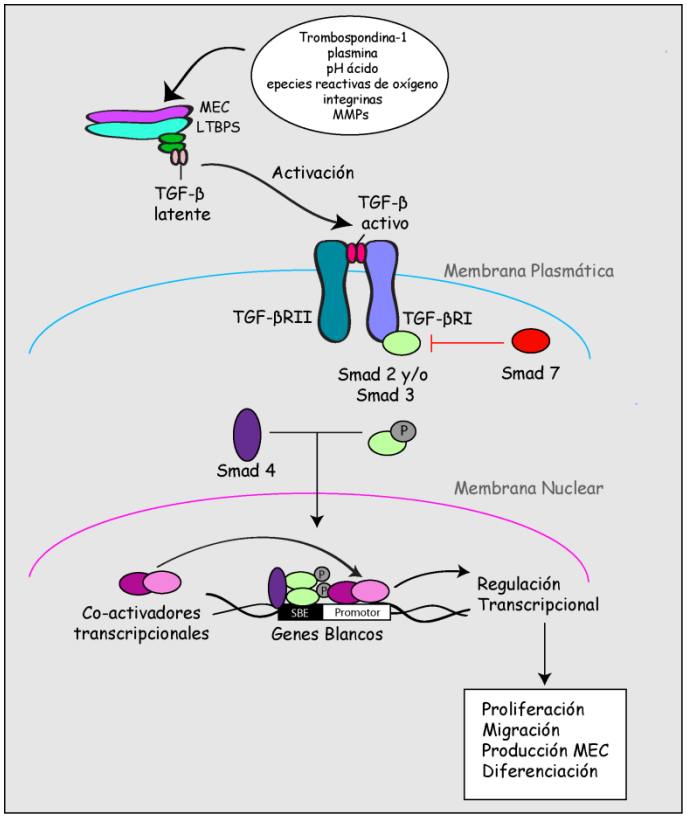

Figura 5: *Vía Canónica de TGF-β y respuestas celulares.*

TGF-β1 activo también puede interaccionar con otros correceptores, como endoglina y betaglicanos, conocidos como receptores TRIIIs[11,221] y además puede activar otras vías intracelulares independientes de la señalización Smad (vía no canónica), como la ruta de MAPKs, NF-κB, RhoA/ROCK, PI3K/Akt o el factor inducible por hipoxia.[155,208,279]

Bajo condiciones normales, la TEM inducida por TGF-β1 es contrarrestada por la proteína BMP-7, que actúa como un antagonista natural de TGF-β1, uniéndose a TRI impidiendo la fosforilación de R-Smad favoreciendo la arquitectura epitelial.[126] Durante un

cuadro inflamatorio persistente, este equilibrio se pierde beneficiando la generación de nuevos fibroblastos, manteniendo de este modo el fenotipo mesenquimático.[164] BMP-7 también es conocida como proteína osteogénica-1 o I-Smad, y es uno de los 20 tipos distintos de BMPs identificados.[1] En varios modelos de daño renal, se ha demostrado que BMP-7 no sólo atenúa la fibrogénesis renal, sino que también restaura la estructura de las unidades epiteliales tubulares.[271] BMP-7 podría tener un efecto renoprotector y posiblemente podría revertir la fibrosis intersticial.[127,270,271]

4. Gremlin

Gremlin es una proteína que pertenece a la familia de antagonistas de las BMPs, esta familia incluye a Cerberus (*head-inducing factor*), DAN (*tumour suppressor*), Chordina, Noggina y Folistatina.[65,79,141,213] Gremlin, también llamado Drm (*Down-regulated by-mos*), fue inicialmente conocido como IHG-2 (*induced in high glucose-2*) ya que fue identificado como uno de los genes del desarrollo inducidos en células mesangiales sometidas a altas concentraciones de glucosa.[138] La deleción de Gremlin en ratones C57BL/6 con fondo genético homogéneo es letal, debido a que provoca agenesis renal completa.[95,148]

En su estructura contiene potenciales SLN cercanos a su extremo carboxilo terminal, sitios de N-glicosilación y sitios de fosforilación.[237] (**Figura 6**) Además de ser una proteína secretada, también puede ser localizada en la superficie externa de la célula y dentro del compartimento retículo endoplásmico-golgi.[237] Gremlin se expresa preferentemente en células terminalmente diferenciadas tales como neuronas y células epiteliales alveolares.[236] Gremlin es capaz de antagonizar los efectos de las BMPs participando en procesos de crecimiento, diferenciación y desarrollo.[79] Durante la nefrogénesis, ejerce su efecto a través de su unión directa y heterodimerización con BMP-2, BMP-4 y BMP-7, interfiriendo con la capacidad de estos ligandos de unirse a sus receptores, regulando de esta manera el desarrollo renal.[105,148,159]

Evidencias recientes sugieren que Gremlin podría ser un importante promotor de fibrosis en diferentes patologías, incluyendo, fibrosis hepática,[15] pulmonar,[161] hipertensión pulmonar e idiopática[26,36] y fibrosis miocárdica.[156] Sorprendentemente, en la fibrosis pulmonar la sobre-expresión de Gremlin induce reversión de fibrosis, mediada por la inhibición de las BMPs e inducción del factor protector FGF10.[54] Estos datos sugieren que son necesarios

estudios adicionales sobre esta molécula en distintas fases del proceso fibrótico y en los órganos implicados. Además, se han descrito una serie de funciones intracelulares de Gremlin, independientes de BMPs, las cuales mediarían respuestas celulares tales como la estimulación de la migración de células endoteliales, a través de su unión al receptor VEGFR2.[150] Por lo tanto, Gremlin es una proteína con múltiples funciones, fisiológicas y patológicas, las cuales se realizan mediante mecanismos dependientes e independientes de BMPs.

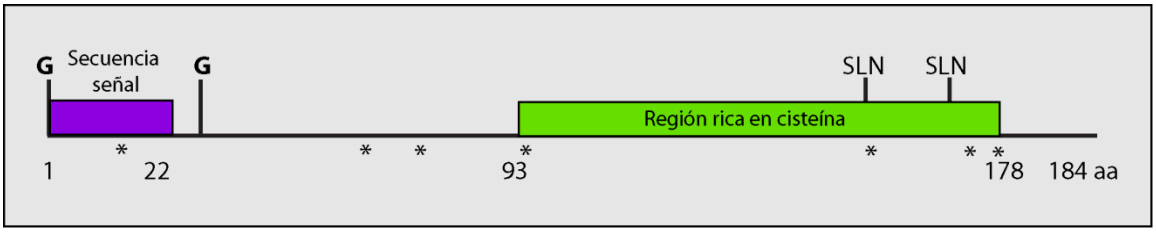

Figura 6: *Secuencia peptídica y modificaciones post-traduccionales de Gremlin. G: sitios de N-glicosilación, * sitios de fosforilación, SLN: secuencia de localización nuclear.*

Muchos estudios, incluidos los de nuestro grupo, sugieren la participación de Gremlin en enfermedades fibróticas crónicas como la nefropatía diabética experimental y humana.[46,54,105,106,136,160,193,243,280]

Gremlin y VEGFs pertenecen a la superfamilia de proteínas nudo de cisteína,[242] esto hizo que el grupo de Mitola investigara si, debido a sus homologías estructurales, Gremlin podía unirse al receptor VEGFR2 e inducir angiogénesis, la principal respuesta del ligando canónico VEGFA.[150,225] (**Figura 7**)

VEGFR2, también conocido como KDR (*kinase insert domain containing receptor,* en humano) o Flk-1 (*Fetal liver kinase-1,* en ratón), participa activamente en migración celular, proliferación, supervivencia y permeabilidad vascular a través de su activación por VEGF.[192] Muchos estudios han demostrado que VEGF fosforila al VEGFR2 en la Tyr951 en células endoteliales durante el desarrollo y en la formación de tumores en la vasculatura.[97] VEGFR2 se expresa en múltiples tipos celulares, tales como células tumorales, progenitores de células endoteliales y células endoteliales maduras.[72] Además se ha descrito que los podocitos no expresan niveles detectables del tránscrito o de la proteína de VEGFR2.[224]

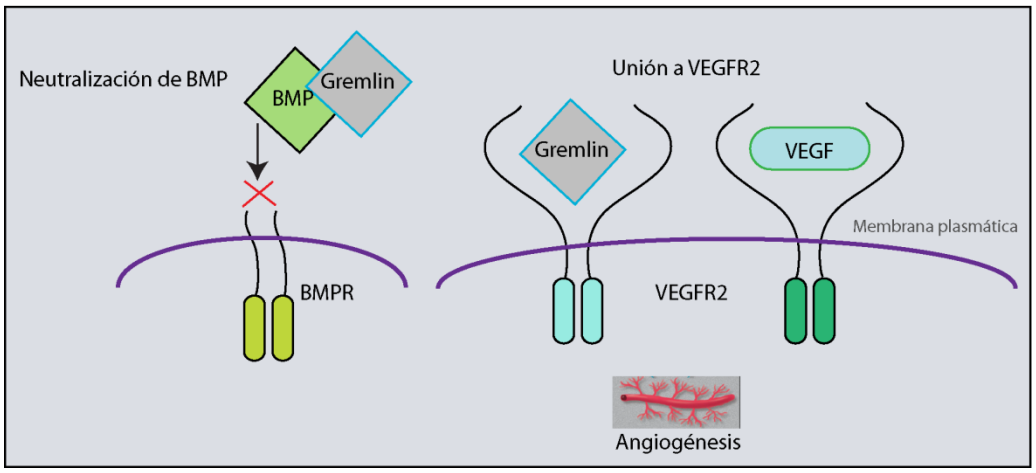

Figura 7: *Esquema de las acciones de Gremlin.*

5. Sistema Renina-Angiotensina-Aldosterona (SRAA)

El SRAA es considerado un sistema con características endocrinas y constituye el principal regulador de la función renal ya que tiene un papel clave en la homeostasis de la presión arterial y el balance de electrolitos. Muchos estudios han demostrado la activación del SRAA en enfermedades renales experimentales y humanas.[200,252,285]

El SRAA está formado por una serie de péptidos generados a partir de Angiotensinógeno por acción de diversas peptidasas. El Angiotensinógeno es liberado en el hígado, que mediante la acción de la renina producida por el aparato yuxtaglomerular del riñón, genera el decapéptido Angiotensina I (Ang I). Ang I es activada por la enzima convertidora de angiotensina (ECA) pulmonar, que es una metaloproteasa dependiente de Zinc que actúa sobre el extremo C-terminal de Ang I, generando el octapéptido Ang II. La Ang II se une a receptores específicos en la corteza adrenal, lo que genera la liberación de Aldosterona. La función principal del SRAA circulante es regular respuestas fisiológicas, manteniendo la presión arterial mediante la vasoconstricción a través de la Ang II y la retención de sodio a nivel del túbulo colector mediado por la Aldosterona. [69,151]

Los estudios realizados en los últimos años han demostrado la existencia de un SRAA que actúa a nivel local y que participa en procesos patológicos como proliferación celular, apoptosis, acumulación de MEC e inflamación y actúa de manera independiente del SRAA sistémico, que regula las respuestas fisiológicas. Se han descrito niveles elevados de SRAA en

patologías crónicas tales como infarto de miocardio, hipertensión arterial y enfermedad renal crónica.[175,196,231] A nivel renal tanto la hiperglicemia como la proteinuria son capaces de activar el SRAA local.[165] Algunos estudios han demostrado niveles elevados de ECA y aumento de producción de Ang II en el riñón en pacientes con diversas nefropatías crónicas como nefropatía diabética y nefropatía membranosa.[143,144] Fármacos que bloquean el SRAA como los inhibidores de la ECA y antagonistas de los receptores AT1 han demostrado mejorar la evolución clínica de pacientes con enfermedades renales y cardiovasculares.[4,24,173,194]

5.1. Angiotensina II

La Ang II, principal péptido efector del SRAA, es considerada un factor de crecimiento que participa en proliferación celular, acumulación de MEC e inflamación,[49,195,250] contribuyendo a la patogenia de enfermedades crónicas como hipertensión, arteriosclerosis, hipertrofia cardiaca y daño renal.[199] Muchos estudios *in vitro* han demostrado que Ang II activa células renales para producir factores pro-fibróticos y proteínas de MEC.[144,200,253] Además regula la proliferación y participa en el reclutamiento de células pro-inflamatorias en el riñón.[199,231,250]

Ang II ejerce sus efectos biológicos mediante la unión a receptores específicos, denominados AT1 y AT2.[42] (**Figura 8**) Ang II a través de su receptor AT1, ejerce la mayoría de sus acciones fisiológicas y fisiopatológicas, tales como vasoconstricción, liberación de Aldosterona y regulación de la MEC.[49,75,238] La activación del receptor AT2 disminuye la presión arterial por liberación de óxido nítrico, inhibe la proliferación celular e induce diferenciación y apoptosis.[199,250]

Ang II activa respuestas intracelulares que participan en la señalización del proceso inflamatorio y fibrótico, como la activación de la ruta de las MAPKs,[62] la producción de ROS y la activación de factores de transcripción como el NF-κB, AP-1 y el factor inducible por hipoxia (HIF-1).[198,209] Ang II es capaz de inducir la síntesis endógena de factores de crecimiento tales como TGF-β y CTGF,[200,250] regular mediadores inflamatorios como IL-6, TNF-α y quimioquinas como MCP-1 [59,196,200,260,281] y péptidos vasoactivos como endotelina-1[77,107] que median algunos de los efectos de Ang II.

Existe una interesante relación entre Ang II y TGF-β en el riñón.[200] En células renales en cultivo, Ang II estimula la expresión de TGF-β, y el bloqueo de TGF-β modifica algunas de las

respuestas de Ang II, entre ellas la regulación de la MEC.[253] Se ha demostrado que bloqueantes de Ang II disminuyen la sobre-producción de TGF-β y la fibrosis renal.[204,253] TGF-β y Ang II comparten mecanismos de señalización implicados en fibrosis, entre los que destaca la vía Smad.[200,253] Nuestro grupo ha descrito que Ang II activa la vía Smad por un mecanismo independiente de TGF-β, contribuyendo a la TEM.[28] Aunque TGF-β es uno de los principales factores que intervienen en la regulación de la fibrosis, las estrategias terapéuticas diseñadas para su bloqueo no han sido muy beneficiosas probablemente por sus propiedades anti-inflamatorias.[63] Sin embargo, los fármacos inhibidores de Ang II han demostrado ser una buena opción para bloquear TGF-β en humanos.

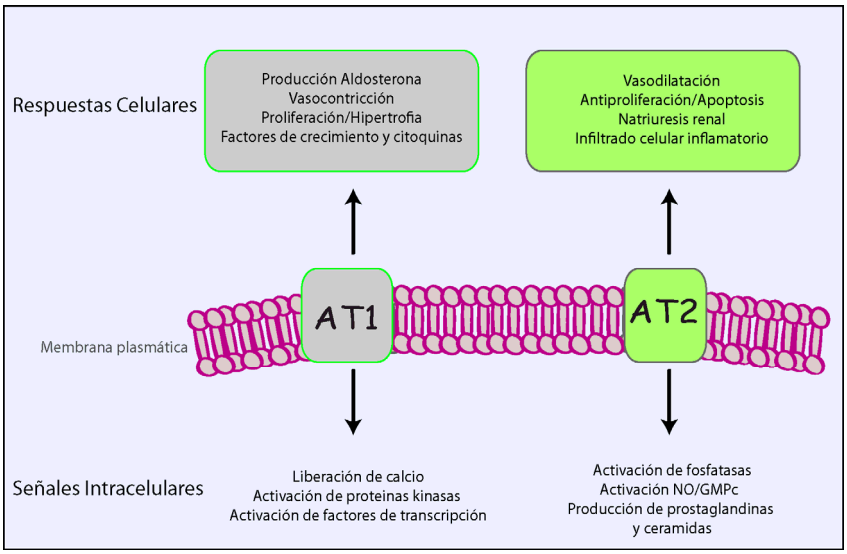

Figura 8: *Respuestas celulares y mecanismos moleculares activados por Ang II a través de sus receptores.*

6. Vía de Señalización Notch

La vía de señalización Notch ha sido altamente conservada durante la evolución y es utilizada por organismos multicelulares para especificar las decisiones del destino celular durante la formación de estructuras complejas, tales como el riñón. Notch influencia críticamente la especificación del destino celular, proliferación, diferenciación, angiogénesis y apoptosis celular, siendo ampliamente investigado por estar implicado en diversas enfermedades neoplásicas y no neoplásicas.[84,103,177,205,257]

La familia de receptores Notch consta de Notch1/2/3/4, son proteínas de transmembrana tipo I y están compuestas de dos subunidades asociadas no covalentemente. Los ligandos de los receptores Notch descritos son: delta-like 1/3/4 y Jagged1/2.[23] El ligando Jagged-1 en su porción extracelular contiene un dominio DSL (iniciales de los nombres de los ligandos Delta y Serrate de *D. melanogaster* y *Lag de C. elegans*) el cual participa en la unión al receptor, posee además un dominio rico en cisteína involucrado en la especificidad de tal unión y repeticiones tipo EGF que la estabilizan.[113] La unión del ligando al receptor Notch promueve dos eventos de corte proteolítico en este último. El primer corte se produce a nivel extracelular, donde se expone el dominio de corte S2, proceso mediado por dos proteasas: ADAM-10 y ADAM-17. Estas proteasas generan una forma activada de Notch que permanece unida a la membrana, la cual es sustrato para la enzima γ-secretasa que cataliza el segundo corte a nivel de la cara interna de la membrana plasmática en el dominio de corte S3.[23,109,110] Este corte libera el dominio intracelular de Notch (NICD), que migra hacia el núcleo donde se asocia con el activador transcripcional, RBP-Jκ (*Recombination Signal-Binding Protein 1 for J-Kappa*) y activa la transcripción del sitio de unión a DNA (GTGGGAA) de RBP-Jκ. En ausencia de NICD, RBP-Jκ se asocia con un complejo correpresor y actúa como un represor transcripcional de su sitio de unión al DNA.[84] Este complejo aumenta la expresión de genes diana primarios de la vía de señalización Notch, tales como *hairy enhancer of split* que incluyen HES1/2/3/4/5/7, y la proteína represora relacionada a HES (HERP)1/2/3; ambas familias son represores transcripcionales.[23,55,56,84] (**Figura 9**)

Durante la embriogénesis renal, el ligando Jagged-1 se expresa en túbulos colectores, agregados pre-tubulares y en células endoteliales del glomérulo en formación, activando de esta manera los receptores Notch-1 y Notch-2, lo que controla el patrón celular en el desarrollo pronéfrico y en la glomérulo-génesis.[32,134,137,181] Esta interacción está involucrada en procesos fisiológicos y patológicos, los que incluyen diferenciación celular endotelial,[10] deterioro celular endotelial,[118] angiogénesis,[286] regeneración de la vasculatura[102,118] y cáncer.[184]

La activación de la vía Notch está prácticamente ausente en el glomérulo de riñones adultos sanos, mientras que su activación se observa en progenitores renales y en podocitos de pacientes con desórdenes glomerulares.[108] Los componentes de la vía Notch se expresan en un amplio rango de enfermedades renales, entre ellas la lesión glomerular mínima, nefropatía

membranosa, nefritis lúpica, nefroesclerosis hipertensiva, glomerulonefritis crescéntica, nefropatía por IgA, nefropatía diabética y glomérulo-esclerosis focal y segmentaria, donde la expresión de Notch-1 en podocitos se correlaciona con albuminuria y glomérulo-esclerosis, mientras que la expresión de Notch-1 activo en túbulos se asocia con fibrosis túbulo-intersticial.[158]

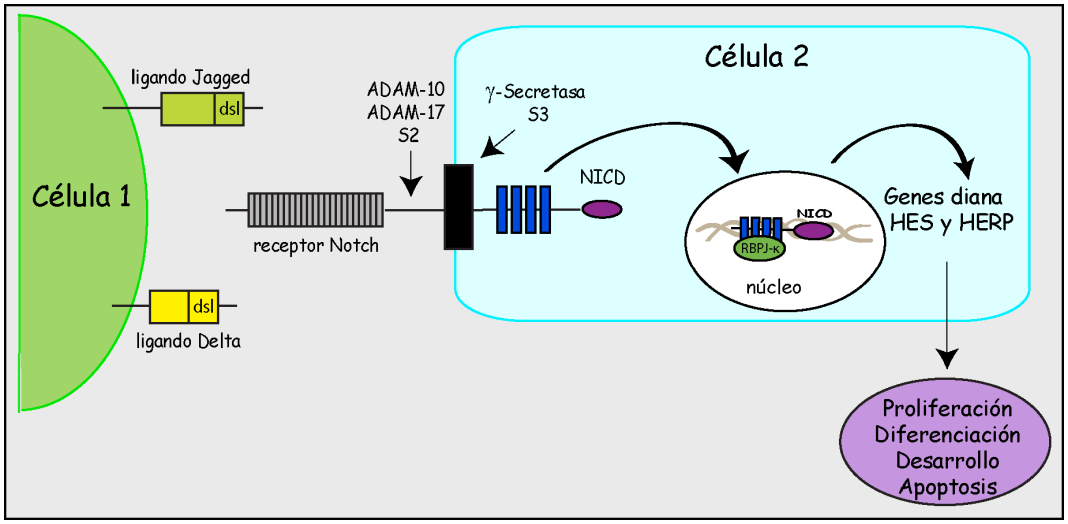

Figura 9: *Vía de señalización Notch y respuestas celulares.*

En modelos experimentales de daño túbulo-intersticial, la activación de la vía Notch se presenta en células tubulares e instersticiales.[153,166,263] Estudios a nivel glomerular en ratones transgénicos que poseen Notch-1 activado específico de podocitos, han demostrado que presentan albuminuria, glomérulo-esclerosis y pérdida de podocitos.[167,247] y en estudios *in vitro*, la expresión de Notch-1 activado en podocitos causa apoptosis.[167] Sin embargo, los efectos beneficiosos de la modulación de Notch en la progresión de la enfermedad renal es aún un tema controvertido.[13,67,108]

La vía de señalización Notch fue originalmente descrita como un mecanismo que participa en la inhibición de la diferenciación celular y que mantiene a las células en un estado indiferenciado, de esta manera permite a las células responder a las señales inductoras en el tiempo apropiado para facilitar la diversificación celular. De hecho, la señalización Notch se caracteriza por direccionar las células hacia un estado de diferenciación alterno y puede bloquear o promover la diferenciación celular, dependiendo del linaje celular.[113]

Mutaciones en receptores y ligandos de Notch llevan a anomalías en muchos tejidos, incluyendo vasos, timo, región craneofacial, extremidades, costillas, sistema nervioso central, corazón, riñón y células hematopoyéticas. Las patologías más estudiadas son el Síndrome de Alagille y la Arteriopatía autosómica-dominante con infartos subcorticales y leucoencefalopatía (CADASIL), donde se observan anomalías de severidad variables, producidas por mutaciones en el receptor Notch-3 y el ligando Jagged-1, respectivamente.[84]

Mediante estudios *in silico* se ha demostrado que los genes Jagged-1 y Hes-1 tienen similitudes significativas con Gremlin, en términos de estructura del promotor y de elementos de unión de microRNA predichos.[245] Sin embargo, no hay estudios sobre el efecto de Gremlin en la ruta Notch. Actualmente hay estudios clínicos utilizando inhibidores de la γ-secretasa, que bloquean esta ruta, en enfermedades como Alzheimer's y leucemia,[99] por lo que también podría ser una nueva diana terapéutica para la enfermedad renal crónica.

II. OBJETIVOS

OBJETIVO GENERAL

La progresión de la enfermedad renal se caracteriza por una respuesta inflamatoria inicial, seguida de fibrosis túbulo-intersticial que conlleva a fallo renal terminal. Previamente nuestro grupo ha descrito que en patología renal humana se reactiva la expresión de Gremlin, un gen de desarrollo que no se expresa en riñón normal adulto y que se desconoce su función. En la enfermedad renal se encuentran activados distintos mecanismos de señalización, el objetivo de esta tesis fue evaluar si Gremlin podría ser un mediador de daño renal capaz de regular distintos procesos implicados en la progresión de la enfermedad renal como son inflamación y fibrosis.

Objetivos específicos

1. Investigar los efectos de Gremlin en riñón en condiciones fisiológicas, el posible receptor implicado (candidato VEGFR2) y sus mecanismos intracelulares asociados. Para ello se utilizará un modelo en ratón de inyección de Gremlin en parénquima renal y estudios *in vitro*.
   1.1 Estudiar si VEGFR2 es un receptor funcional de Gremlin en riñón.
   1.2 Estudiar los efectos de Gremlin en riñón evaluando si regula procesos inflamatorios y los potenciales mecanismos de señalización implicados.
   1.3 Evaluar la intervención directa de Gremlin en procesos fibróticos y si es un mediador de las acciones pro-fibróticas de TGF-β.
2. Determinar la activación de la ruta Gremlin/VEGFR2 en modelos experimentales de daño renal y su posible modificación terapéutica.
3. Investigar si Gremlin y Ang II modulan la vía de señalización Notch y su implicación en el daño renal experimental y humano.

III. MÉTODOS Y RESULTADOS

1. El eje Gremlin/VEGFR2 como un nuevo abordaje terapéutico para la enfermedad renal

Gremlin tiene un papel importante en nefrogénesis.[73,95,148] Sin embargo, su rol en la patogenia del daño renal no está bien estudiado. En riñón adulto sano Gremlin no se expresa, pero se induce en enfermedades renales crónicas.[28,46,147,160] Gremlin antagoniza los efectos de las BMPs por unión directa y heterodimerización, impidiendo que estas proteínas se unan a sus receptores.[79,105,148] Recientemente se ha descrito que Gremlin es un nuevo ligando no canónico del receptor VEGFR2, implicado en la angiogénesis en células endoteliales, proceso independiente de la señalización de BMPs.[150] El primer objetivo de este trabajo fue evaluar si Gremlin puede unirse y activar el receptor VEGFR2 en células renales y su relación con las respuestas de Gremlin en el riñón.

En este trabajo se demuestra por primera vez que Gremlin se une y activa el VEGFR2 en riñón y en células renales en cultivo, de forma independiente de BMPs. Utilizando un modelo experimental de administración intrarrenal de Gremlin en ratones sanos, hemos observado que se activa la ruta del VEGFR2 de forma rápida y sostenida, principalmente localizada en células túbulo-epiteliales. Gremlin induce una respuesta inflamatoria en el riñón, produciéndose una rápida activación del factor NF-κB, esta respuesta se caracteriza por la infiltración de monocitos/macrófagos (F4/80) y linfocitos (CD3), y aumento en la expresión renal de factores pro-inflamatorios (MCP-1, Rantes e IL-6) y de biomarcadores de daño renal (NGAL y KIM-1). El bloqueo de la señalización de VEGFR2 mediante el tratamiento farmacológico utilizando el inhibidor de la quinasa del VEGFR2, SU5416, demostró que la respuesta inflamatoria inducida *in vivo* por Gremlin esta mediada por este receptor.

En los estudios *in vitro*, mediante inhibidores farmacológicos y silenciamiento génico, hemos observado que Gremlin regula la vía NF-κB/genes inflamatorios, a través del receptor VEGFR2. Como el aumento de Gremlin se asoció a daño renal y a la activación de VEGFR2, nuestro segundo objetivo fue evaluar la modulación terapeútica de esta ruta. En el modelo de daño renal experimental por obstrucción unilateral ureteral, en el modelo de infusión de Ang II y en biopsias renales de pacientes con diversas nefropatías, se induce la re-expresión renal de Gremlin asociado a la activación del VEGFR2. Además, el bloqueo de la ruta del VEGFR2 disminuyó el infiltrado inflamatorio renal en el modelo experimental de daño renal por UUO.

Los resultados presentados en este estudio demuestran que Gremlin tiene un papel importante en la regulación de la inflamación renal mediante unión y activación del VEGFR2 y la modulación de la vía canónica del NF-κB, lo que sugiere a Gremlin como una diana terapéutica potencial para enfermedades renales inflamatorias.

Title page

The axis Gremlin/VEGFR2 as a new therapeutic approach for renal diseases.

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Running title: Gremlin involved in inflammation in the kidney

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Key words: Gremlin, fibrosis, VEGFR2, NF-κB.

ABSTRACT

Gremlin is a developmental gene up regulated in human chronic kidney diseases and proposed as a potential mediator of experimental renal damage. In cultured renal cells Gremlin regulates cell proliferation and fibrosis, but there are no studies evaluating Gremlin actions in the kidney. Gremlin binds to bone morphogenetic proteins (BMPs) and acts as an antagonist regulating among other process nephrogenesis and fibrosis. In cultured endothelial cells Gremlin binds to vascular endothelial growth factor receptor-2 (VEGFR2) to induce angiogenesis. Our aim was to investigate the direct effects of Gremlin in the kidney, evaluating the receptor and downstream mechanisms involved. In cultured tubular epithelial cells, Gremlin binds to VEGFR2 and activates this signaling pathway independent of BMPs. Administration of recombinant Gremlin into the mice induced a rapid and sustained activation of VEGFR2 signaling, located in proximal tubular epithelial cells. Gremlin caused an inflammatory response in the kidney, characterized by renal activation of the nuclear factor-κB pathway, up-regulation of pro-inflammatory factors (chemokines, adhesion molecules and cytokines) and infiltration of monocytes/macrophages and lymphocytes. Treatment with the VEGFR2 kinase inhibitor SU5416 diminished Gremlin-induced renal inflammatory responses. Moreover, in cultured tubular epithelial cells VEGFR2 blockade, by kinase inhibition or gene silencing, inhibited Gremlin-mediated NF-κB activation and induction of proinflammatory genes. In the mice model of renal damage by unilateral ureteral obstruction, Gremlin overexpression was associated to VEGFR2 pathway activation, and VEGFR2 kinase inhibition diminished renal inflammation. These data demonstrate that Gremlin activates VEGFR2 signaling pathway in the kidney linked to renal inflammation.

Keywords: Gremlin, VEGFR2, NF-κB, inflammation

INTRODUCTION

Gremlin is a member of cysteine knot protein family that includes transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMP).[1] Gremlin has been highly conserved during evolution and has an important role in embryogenesis.[2,3] However, the functions of Gremlin in adult tissues in normal and pathological conditions are not fully described.

Gremlin binds to BMP-2, BMP-4, and BMP-7, forming heterodimers that prevents BMP/ligand-receptor interaction and subsequent downstream signaling.[4] This function of Gremlin as a BMP antagonist plays a critical role in axial patterning activity and nephrogenesis,[3,5] regulates diverse processes including growth, differentiation and development,[3] and has been involved in cancer, glaucoma and fibrotic processes, including pulmonary and liver fibrosis.[6-10] Several studies suggest that Gremlin also exerts direct cellular actions, independent of BMP antagonism, such its ability to suppress tumor-genesis and to stimulate migration of endothelial cells.[11,12] Vascular endothelial growth factor receptor-2 (VEGFR2) is a tyrosine kinase receptor that can be activated by different members of the VEGF family[13] and, as recently described in cultured endothelial cells, by Gremlin.[14-16] Previous studies have clearly demonstrate that VEGF/VEGFR2 is tightly linked to angiogenesis, both in physiology and pathology, such as in the growth of cancer.[14,17] However, the biology of Gremlin/VEGFR2 is not obvious and *in vivo* studies remain to be performed.

Gremlin has emerged as a novel modulator of diabetic nephropathy. In experimental diabetes and in patients with diabetic nephropathy renal Gremlin overexpression has been described,[18,19,20] and GREM1 variant rs1129456 was associated with diabetic nephropathy.[21] In the experimental model of streptozotocin-induced diabetes, Gremlin deletion, by grem1 heterozygous knockout mice or gene silencing ameliorated renal damage.[22,23] In renal cells, Gremlin regulates cell proliferation and fibrotic-related events.[23,24,25] In addition, Gremlin gene silencing diminished TGF-β induced profibrotic events[24] and apoptosis.[25] These experimental data suggest that Gremlin blockade could be potential therapeutic target for renal diseases. However, there are no studies evaluating the direct effects of Gremlin in the kidney. In this paper we have investigated whether Gremlin could activate the VEGFR2 signaling pathway in the kidney and subsequent renal responses.

RESULTS

Gremlin binds to VEGFR2 and activates its downstream signaling in cultured tubular epithelial cells.

Our first aim was to evaluate whether Gremlin could bind to VEGFR2 and activate its downstream signals in cultured renal cells, as observed in human endothelial cells.[14] Live-cell imaging was done by confocal time-lapse microscopy to visualize Gremlin binding to tubular epithelial cells. After adding labeled Cy5-Gremlin to HK2 cells the red immunofluorescent signal was rapidly located at the cellular membrane (figure 1A). The involvement of VEGFR2 was evaluated by gene silencing. HK2 cells were transfected with a siRNA against VEGFR2 or its corresponding scrambled control siRNA (both Cy3-labeled), stimulated with Cy5-Gremlin and binding was evaluated by confocal microscopy. In control siRNA-transfected HK2 cells, Cy5-Gremlin positive immunofluorescent binding signal was found, whereas in VEGFR2-silenced cells the signal was decreased (Figure 1B).

To investigate further whether Gremlin directly interacts with VEGFR2, cells were incubated with Gremlin for 10 minutes, following by a cross-linking procedure to fix the proteins anchored to the cell surface. Then, total proteins were isolated and immunoprecipitated with an anti-VEGFR2 antibody. Covalently-linked Gremlin complexes were then analyzed by PAGE and Western blotting with an anti-Gremlin antibody. In Gremlin-treated cells, but not in untreated ones, VEGFR2 complexes were found (Figure 1D), demonstrating that Gremlin interacts with VEGFR2 in tubular epithelial cells.

One of the earliest steps on VEGFR2 activation is auto-phosphorylation on tyrosine residues.[13] In human tubular epithelial cells, incubation with Gremlin for 10 min increased VEGFR2 phosphorylation (p-Tyr VEGFR2 996), showing a positive dose-response between 10 to 50 ng/ml (Figure 2A). Gremlin activated VEGFR2 as early as at 5 min, remained elevated up to 48 hours (Figure 2B). Moreover, colocalization of Cy5-Gremlin binding and phosphorylated-VEGFR2 was found (Figure 1C). These data clearly show that Gremlin binds to VEGFR2 and activates its downstream signaling pathway in cultured tubular epithelial cells.

BMPs are not involved in VEGFR2 activation caused by Gremlin

Some Gremlin actions are mediated by its effect as a BMPs antagonist.[3] In HK2 cells, Gremlin-induced VEGFR2 phosphorylation was not modified in the presence of BMP-2, BMP-4 or BMP-7 (Figure 2C). These data demonstrate that Gremlin directly activates VEGFR2 pathway, independent of BMPs antagonism, in cultured renal cells.

Gremlin activates VEGFR2 signaling pathway in the kidney.

To investigate the *in vivo* effect of Gremlin in the kidney, a model of intra-renal injection of Gremlin in C57Bl/6 mice was performed. First, localization of Gremlin binding to renal structures was done by confocal microscopy. For these experiments, labeled Cy5-Gremlin was injected in the renal parenchyma of the left kidney. In Cy5-Gremlin-injected kidneys a red immunofluorescent signal was

observed, with a maximal intensity at 15 min, mainly located in tubular cells (Figure 3A). No red signal was found in saline-injected kidneys (data not shown).

Next, we studied whether Gremlin could activate VEGFR2 signaling. In Gremlin-injected kidneys increased renal levels of phosphorylated VEGFR2, compared to its corresponding contralateral kidney, were found (Figure 3B and C). VEGFR2 activation was observed at 15 min, decreasing thereafter, and presenting a late activation after 24-48 hours (Figure 3B and C), Immunofluorescence revealed that Gremlin activated VEGFR2 mainly in proximal tubular cells *in vivo* (Figure 3D).

Gremlin induces interstitial inflammatory cell infiltration in the kidney via VEGFR2.

Administration of Gremlin *in vivo* induced an inflammatory response in the kidney after 48 hours. Gremlin-injected mice presented an elevated number of monocytes/macrophages ($F4/80^+$ cells) and T-lymphocytes ($CD3^+$ cells) in the kidney, compared to control mice (Figure 4A and B). To evaluate further renal damage, two novel described biomarkers, KIM-1, related to the transition from acute to chronic renal damage,[26,27] and NGAL, an early marker of kidney disease,[28] were determined. In Gremlin-injected kidneys, KIM-1 and NGAL mRNA expression were markedly upregulated (Figure 4C). The renal inflammatory infiltrate is regulated by local induction of chemotactic factors.[29] Gremlin increased the renal expression of several pro-inflammatory factors, including chemokines and cytokines (Figure 4D and E). Interestingly, VEGFA gene expression was not modified in response to Gremlin administration (Figure 4D).

To evaluate whether VEGFR2 pathway activation is involved in Gremlin-mediated effects, mice were treated with SU5416, a specific VEGFR2 kinase inhibitor.[30] Treatment with SU5416 blocked VEGFR2 activation, as shown by immunohistochemistry (Figure 4A and B) and western blot (not shown), and markedly diminished the above-described renal effects of Gremlin, including inflammatory cell infiltration and upregulation of biomarkers of renal damage and proinflammatory factors (Figure 4).

To demonstrate that Gremlin could directly elicit inflammatory-related responses in renal cells, *in vitro* studies were done. In HK2 cells, incubation with Gremlin increased gene expression of proinflammatory factors, starting at 3 hours and remaining elevated until 24 hours (figure 5). The pharmacological blockade of VEGFR2 diminished Gremlin-induced upregulation of proinflammatory genes (figure 5A). Moreover, VEGFR2 gene silencing experiments were done to clearly demonstrate that Gremlin regulates inflammatory factors via VEGFR2 signaling pathway. In control siRNA-transfected HK2 cells, but not in VEGFR2 silenced cells, Gremlin upregulated proinflammatory gene expression (Figure 5B).

Gremlin activates the NF-κB pathway in the kidney

NF-κB activation is an important intracellular signaling pathway involved in renal inflammation.[31] Activation of the canonical NF-κB pathway involves the phosphorylation of the inhibitory Iκ-Bα subunit, dissociation from the complex and its subsequent proteasome-mediated degradation in the cytosol.[32] In cultured tubular epithelial cells, Gremlin increased Iκ-Bα phosphorylation at Ser32 at 5 min, presenting a

dual activation at 48 hours (figure 6A). Phosphorylation of the NF-κB p65 subunit at Ser536 is one of the earliest events in the activation of the canonical NF-κB pathway, and has been related to inducible pro-inflammatory gene up-regulation.[33] In HK2 cells, Gremlin increased p65 phosphorylation as early as 5 min, remained elevated up to 48 hours (Figure 6A). We visualized the NF-κB translocation to the nucleus by confocal microscopy immunocytochemistry. In control cells a diffuse cytoplasmic immunofluorescence was observed with an antibody against the p65 subunit NF-κB, while Gremlin treatment for 30 minutes led to an intense nuclear fluorescence (Figure 6C). The role of VEGFR2 in Gremlin-induced NF-κB activation was demonstrated by the VEGFR2 kinase inhibitor and by gene silencing, evaluating p65 phosphorylation and its nuclear translocation (Figure 6B,C,D and E). The active NF-κB complex is translocated into the nucleus where it binds to specific DNA sequences to regulate gene transcription.[32] Incubation with Gremlin for 1 hour increased p65 DNA binding activity, which was diminished by the VEGFR2 kinase inhibitor (Figure 6F).

Administration of Gremlin into mice kidney induced a rapid activation of the NF-κB pathway. After 15 min, elevated cytosolic phosphorylated IκBα levels were found in Gremlin-injected kidneys compared to contralateral ones, remaining elevated until 48 hours (Figure 7A and 7B). Gremlin also increased nuclear p65 DNA-binding activity (Figure 7C), with a similar kinetics. Treatment with the VEGFR2 kinase inhibitor SU5416 prevented Gremlin-induced NF-κB activation (Figure 7C).

Finally, to evaluate the *in vivo* contribution of NF-κB activation to Gremlin-induced renal inflammation, mice were treated with parthenolide, a specific NF-κB inhibitor[34] Parthenolide abrogated Gremlin-induced renal inflammation, as shown by a significant diminution in the number of inflammatory infiltrating cells (Figures 8A and B) and downregulation of proinflammatory gene expression to control levels (Figure 8C).

Evaluation of the axis Gremlin/VEGFR2 in an experimental model of renal damage

The unilateral ureteral obstruction (UUO) model is characterized by interstitial inflammatory cell infiltration, apoptosis and fibrosis.[34,35] After 5 days of UUO, renal activation of VEGFR2 signaling was found in obstructed kidneys compared to contralateral ones, as determined by increased levels of VEGFR2 phosphorylation mainly located in proximal tubular epithelial cells (figure 9A,B,C and D). In obstructed kidneys, renal overexpression of Gremlin was observed, at protein and gene levels (figure 9A, B and E). In contrast, VEGFA gene expression was not changed in response to renal injury, at least at this time point (Figure 9E). UUO mice presented a renal inflammatory response, characterized by increased interstitial infiltration of monocytes/macrophages and lymphocytes and upregulation of proinflammatory genes. These inflammatory events were marked decreased by the VEGFR2 kinase inhibitor SU5416 (Figure 9A and B). Moreover, biomarkers of renal damage were also downregulated by VEGFR2 signaling blockade. These data show that VEGFR2 blockade diminished renal damage caused by UUO.

Furthermore, to investigate relation Gremlin/VEGFR2 in a model of systemic infusion of Ang II into rats was used. The onset of renal fibrosis in this model is well characterized.[36] Renal levels of

Gremlin was upregulated in response to AngII infusion for 2 weeks compare to saline infused ones, used as controls (Figure 10A). By immunohistochemistry, we have confirmed Gremlin and phosphorylated VEGFR2 renal expression, both expressed mainly in tubule ephitelial cells (Figure 10B and C).

Evaluation of the axis Gremlin/VEGFR2 in human chronic renal disease.

To evaluate whether Gremlin/VEGFR2 activation could be involved in human renal diseases, we evaluated in 8 cases of several nephropathies. By immunohistochemistry, colocalization of phosphorylated-VEGFR2 and Gremlin expression was observed (Figure 11). These data show that in human progressive renal disease activation of Gremlin/VEGFR2 was found.

DISCUSSION

The main finding of our work is that Gremlin binds to and activates VEGFR2 signalling pathway in the kidney linked to renal inflammation. Our *in vitro* studies, clearly demonstrate that in tubular epithelial cells Gremlin regulates proinflammatory factors by VEGFR2 signalling pathway activation. These data suggest that Gremlin could contribute to renal inflammation, and suggest that the axis Gremlin/VEGFR2 could be a potential therapeutic target for inflammatory progressive chronic kidney diseases.

In a Bioacore-based study, the direct interaction of Gremlin and soluble VEGFR2 has been described.[14] In endothelial cells, Gremlin induces angiogenesis mediated by binding to VEGFR2,[14] but differs to canonical heparin-binding VEGFR2 ligands, since Gremlin interacts with heparan sulfate proteoglycan but not with neuropilin-1.[15] Our *in vitro* studies show that Gremlin binds to VEGFR2 in cultured tubular epithelial cells, as demonstrated by confocal time-lapse microscopy to visualize Gremlin binding to the cell in real time, gene silencing and immunoprecipitation studies. After ligand binding to VEGFR2, tyrosine phosphorylation occurs.[13,37,38] In tubular epithelial cells Gremlin rapidly increased VEGFR2 phosphorylation. Moreover, administration of recombinant Gremlin in normal healthy mice kidney induced a rapid and sustained activation of VEGFR2 signalling, mainly located in proximal tubular epithelial cells. Interestingly, Gremlin binding *in vivo* was also located in these cells, showing a colocalization with phosphorylated-VEGFR2. These data suggest that VEGFR2 is a functional Gremlin receptor in the kidney.

In different physiopathological conditions Gremlin could exert both BMP-dependent and BMP-independent functions. Acting as a BMP antagonist, Gremlin affects different processes during growth, differentiation, and development.[3,4,5] In contrast, many *in vitro* studies support BMP-independent Gremlin effects in the regulation of several cellular processes, including angiogenesis, migration, fibrosis and activation of several intracellular signals.[11,14,39-41] These BMP-independent responses could be mediated by a direct activation of VEGFR2 signaling, as we have shown here in cultured tubular epithelial cells, by the lack effect of BMP-2, -4 and -7 on Gremlin-induced VEGFR2 phosphorylation. Interestingly, studies in

cancer cell lines have shown that Gremlin causes cell migration, invasion, and proliferation in a BMP- and VEGFR2-independent manner, suggesting that other receptors can be involved in oncogenic-responses to Gremlin.[12]

The role of Gremlin in the inflammatory/immune response is poorly understood. In an early study Gremlin was described as an inhibitor of monocyte chemotaxis *in vitro*.[40] Posterior studies have demonstrated that, Gremlin binds to macrophage migration inhibitory factor (MIF) in monocyte/macrophage and act as an endogenous MIF antagonist to regulate monocyte migration.[43] However, the relation of Gremlin/monocytes *in vivo* is controversial. In one study of experimental atherosclerosis in ApoE knockout mice, Gremlin administration reduces the content of macrophages in atherosclerotic plaques and attenuated atheroprogression.[42] However, in another study using this model and siRNAs against BMPs, Gremlin and BMP receptor II, have found that under pro-atherogenic conditions BMP signalling prevails, favouring monocyte recruitment and inflammation.[44] A recent study shows that in cultured endothelial cells Gremlin upregulates the expression of various chemokines and cell adhesion molecules.[17] In cancer cell lines, Gremlin also induces proinflammatory/proangiogenic responses *in vivo*.[17] We have found that local delivery of Gremlin induced an inflammatory response in the kidney, characterized by renal gene overexpression of proinflammatory factors (chemokines, adhesion molecules and cytokines) and infiltration of inflammatory cells into the kidney observed at 48 hours. The blockade of VEGFR2 signalling, by treatment with the VEGFR2 kinase inhibitor SU5416, inhibited Gremlin-induced renal inflammation.

One of the earliest downstream responses to Gremlin/VEGFR2 signalling is the activation of the NF-κB pathway, observed as early as 5 min after Gremlin renal-injection. Our *in vitro* studies, using a pharmacological inhibitor of VEGFR2 kinase or gene silencing, demonstrated that Gremlin via VEGFR2 activates the NF-κB pathway and upregulates several proinflammatory genes under NF-κB control. Moreover, Gremlin-induced *in vivo* renal inflammation was prevented when the NF-κB pathway was blocked using the NF-κB inhibitor parthenolide. In experimental and human kidney diseases elevated renal NF-κB activity correlates with upregulation of proinflammatory parameters,[30] as we have reported in human diabetic nephropathy.[44] Although there are no current data in humans, studies done in many experimental models have demonstrated that NF-κB blockade by different approaches, including IκB overexpression, NF-κB decoy oligonucleotides and NF-κB inhibitors, attenuates renal inflammation and ameliorates disease progression.[45-50] Our results suggest that Gremlin binds to and activates VEGFR2 signalling in the kidney, mainly in tubular epithelial cells, linked to activation of the NF-κB pathway and downstream proinflammatory factors upregulation, leading to the recruitment of inflammatory cells into the kidney.

Several *in vitro* studies have investigated the intracellular mechanism elicited upon Gremlin stimulation. Most of the studies have been done in cultured endothelial cells, and include increased phosphorylation of proteins, production of reactive oxygen species, cyclic adenosine monophosphate production and activation of transcription factors, including cyclic adenosine monophosphate-response element (CRE)-binding protein and NF-κB.[17,51] In other cell types, Gremlin stimulates matrix production through the activation of ERK or TGF-β/Smad signalling.[52,53,24] Our *in vivo* and *in vitro* data shows that one of the earliest mechanism activated by Gremlin is the NF-κB pathway that upregulates proinflammatory factors.

In several chronic kidney diseases upregulation of Gremlin has been described.[19,20,54-56] Interestingly, in models of renal damage (unilateral ureteral obstruction and systemic infusion of Ang II) and in patients with different nephropathies Gremlin overexpression was associated to VEGFR2 pathway activation. Moreover, in the UUO model treatment with the VEGFR2 kinase inhibitor diminished renal inflammation. These data suggest that Gremlin overexpression observed during renal injury could contribute to the activation of the renal inflammatory response.

In spite the intense research done the role of VEGF in renal disease progression is not clearly demonstrated. Dependent on timing and predominant pathology, VEGF could exert benefitial or detrimental effects, by inducing neoangiogenesis and hypoxia that if defective can exaggerates injury.[57] Previous studies in the UUO model have described an early VEGFA upregulation, observed at 24 hours, that was downregulated thereafter.[58,59] The reduction of VEGFA levels in UUO could be the result of multiple factors in addition to hypoxia and HIF-1 expression.[60] Accordingly, after 5 days of UUO, VEGFA gene levels were not upregulated in obstructed kidneys compared to contralateral ones. Studies of VEGFA inhibition in rodent models of diabetic nephropathy have generated mixed results, with some studies demonstrating protection from progression and others failing to show benefit,[61-64] unfortunately in those models Gremlin expression was not evaluated. Now, we have observed that in UUO model, VEGFR2 kinase inhibition diminished renal Gremlin overexpression, downregulated inflammatory markes, the number of infiltrating cells and ameliorated renal damage in the experimental model of UUO. Overexpression of soluble VEGFR2 also attenuated fibrosis in UUO model.[58] These data suggest that VEGFR2 blockade could be an interesting therapeutic target for renal diseases.

Several authors have suggested that Gremlin could be considered as a mediator of renal injury in diabetic nephropathy. Experimental studies in the streptozotocin-induced model of type 1 diabetes using grem1 heterozygotus knockout mice or by Gremlin gene silencing have shown attenuated renal lesions,[22,23] mainly by inhibiting proliferation and fibrosis, although inflammatory factors have not been evaluated. In human biopsies of diabetic nephropathy we have observed Gremlin overexpression, mainly in patients with severe renal damage and tubulointerstitial fibrosis.[20] Now, colocalization of Gremlin and

activation of VEGFR2 was found in human diabetic nephropathy, although the functional consequences remains to be determined. Previous studies have described VEGFA upregulation in renal biopsies and plasma from patients with type 1 or 2 diabetes,[65,66] leading to the hypothesis that the increased level of VEGFA in diabetes is detrimental to glomerular function. A transgenic mice model of specific VEGFA overexpression in podocytes presented similar features to diabetic nephropathy, such as a thickened glomerular basement membrane and proteinuria.[67-69] Interestingly, autocrine actions of the podocyte VEGF system extend beyond those of the VEGFA isoform, involving VEGFRs. The VEGFR1/sFLT1 regulates podocyte morphology, by binding to lipid microdomains, and contributes to preserve renal homeostasis,[70] while VEGFR2, transcript or protein, is not detected in podocytes *in vivo*.[67] Rats injected with VEGFR1/sFLT1 soluble receptor develop hypertension, endotheliosis and proteinuria, similar to the lesion observed in podocyte-specific haploinsufficient VEGF mice.[71] Importantly, in human progressive nephropathies we have found that Gremlin overexpression and VEGFR2 activation was located in tubulointerstital cells, clearly showing a different spatial distribution of VEFGA and Gremlin expression pattern, and probably functional consequences.

Perspectives

Chronic kidney disease (CKD) is a major health problem that has reached epidemic proportions and it may lead to end-stage renal disease or early cardiovascular death.[72,73] Moreover, the increasing incidence of diabetes, hypertension and obesity will raise the number of patients with CKD in the near future. Available clinical treatments for CKD only retard disease progression. Our findings indicate that Gremlin binds to VEGFR2 leading to inflammation in the kidney, and identify Gremlin/VEGFR2 as a new therapeutic target for kidney inflammation.

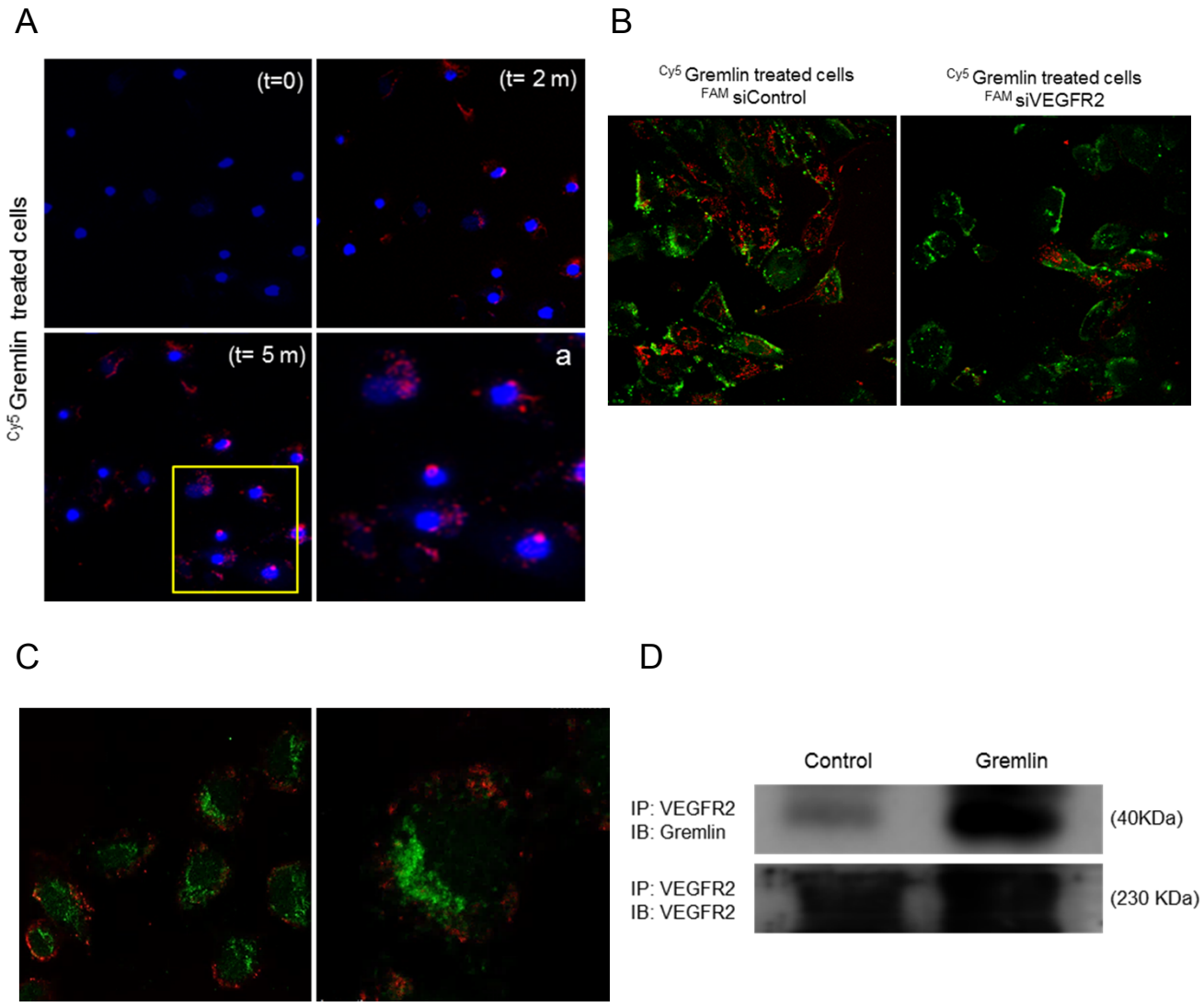

Figure 1. Gremlin binds to VEGFR2 in human tubular epithelial cells. (A) Fluorescent labeled Cy5-Gremlin (100 ng/ml, red staining) was added to HK2 cells and live confocal microscopy images were taken once every 1.3 sec for a period of 5 min. Nuclei stained with DAPI (blue). A magnification is shown in the right (a). **(B)** HK2 cells were transfected with a siRNA against VEGFR2 or its corresponding control siRNA, both FAM-labeled (green) and then stimulated with Cy5-Gremlin for 5 min. **(C)** In Cy5-Gremlin-treated cells, that presented a red membrane immunostaining, colocalization with phosphorylated VEGFR2 (p-VEGFR2) was found. p-VEGFR2 was immunodetected by a secondary AlexaFluor488 labeled antibody (green). Figure shows on the right a magnification (20x) of the image. **(D) Gremlin-VEGFR2 complexes were found by coprecipitation experiments.** Serum-starved HK2 cells were stimulated with 10 ng/ml Gremlin for 10 min. Then, cells were treated with a cross-linker as described in Methods. Cell lysates were immunoprecipitated (IP) with an anti-VEGFR2 and then analyzed by Western Blot (IB) with anti-Gremlin antibody, to determine the complexes formed. Figures show a representative experiment of 3 done.

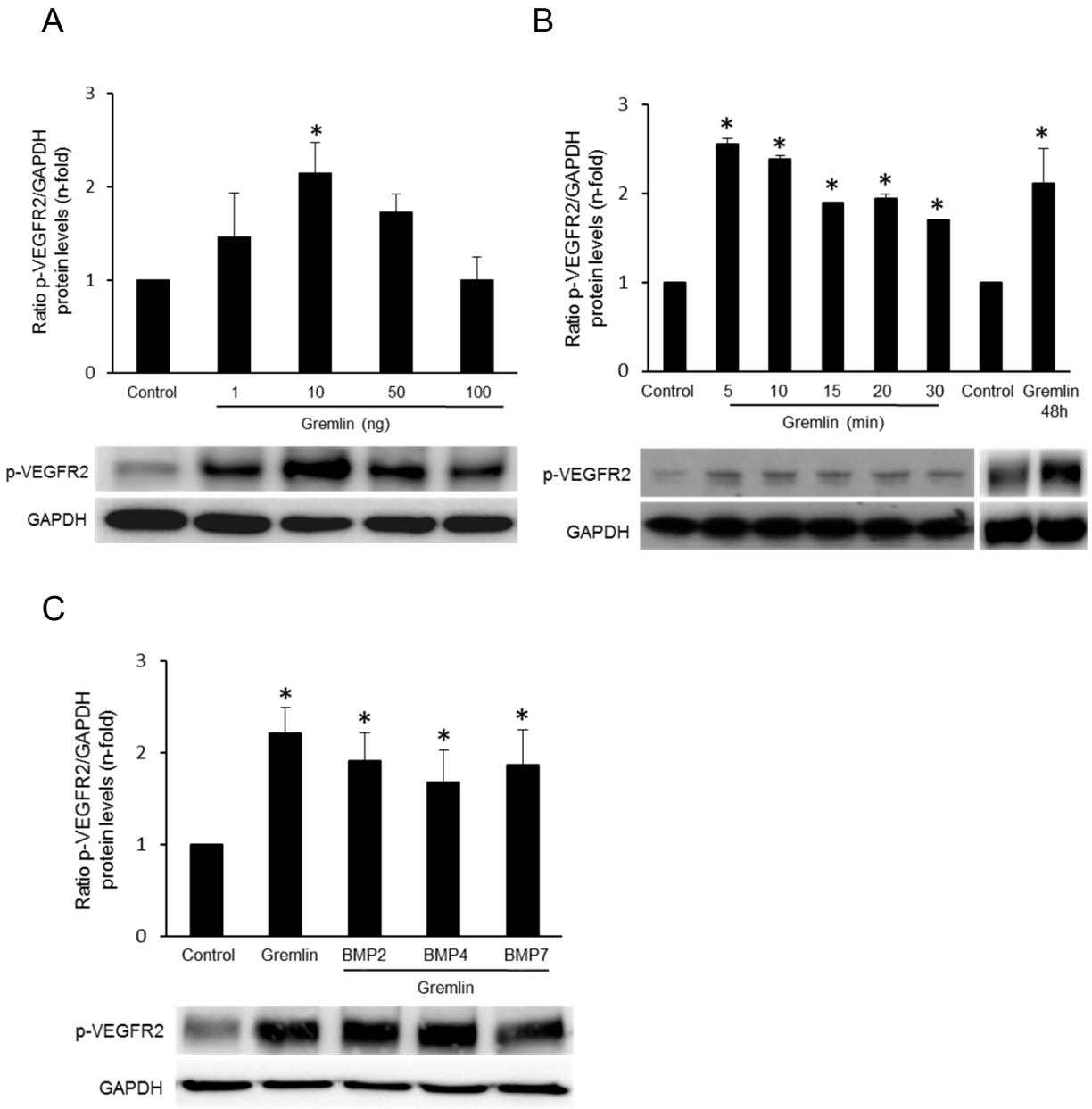

Figure 2. Gremlin activates VEGFR2 signaling, independent of BMPs, in human tubular epithelial cells. HK2 cells were treated with **(A)** several concentrations of Gremlin (range 1-100 ng/ml) for 10 min or with **(B)** 10 ng/ml Gremlin for increasing time periods. VEGFR2 phosphorylated levels (p-VEGFR2) evaluated by western blot were used for determining VEGFR2 pathway activation. **(C)** Cells were preincubated with BMP-2, BMP-4 or BMP-7 at 10 µg/ml for 1 hour before stimulation with Gremlin for 10 min. Results of p-VEGFR2 protein expression levels were obtained from densitometric analysis, as ratio vs its corresponding GAPDH values, and expressed as n-fold over control (considered 1). Figures show a representative western blot experiment and data as mean±SEM of 5 independent experiments. *p<0.05 vs control.

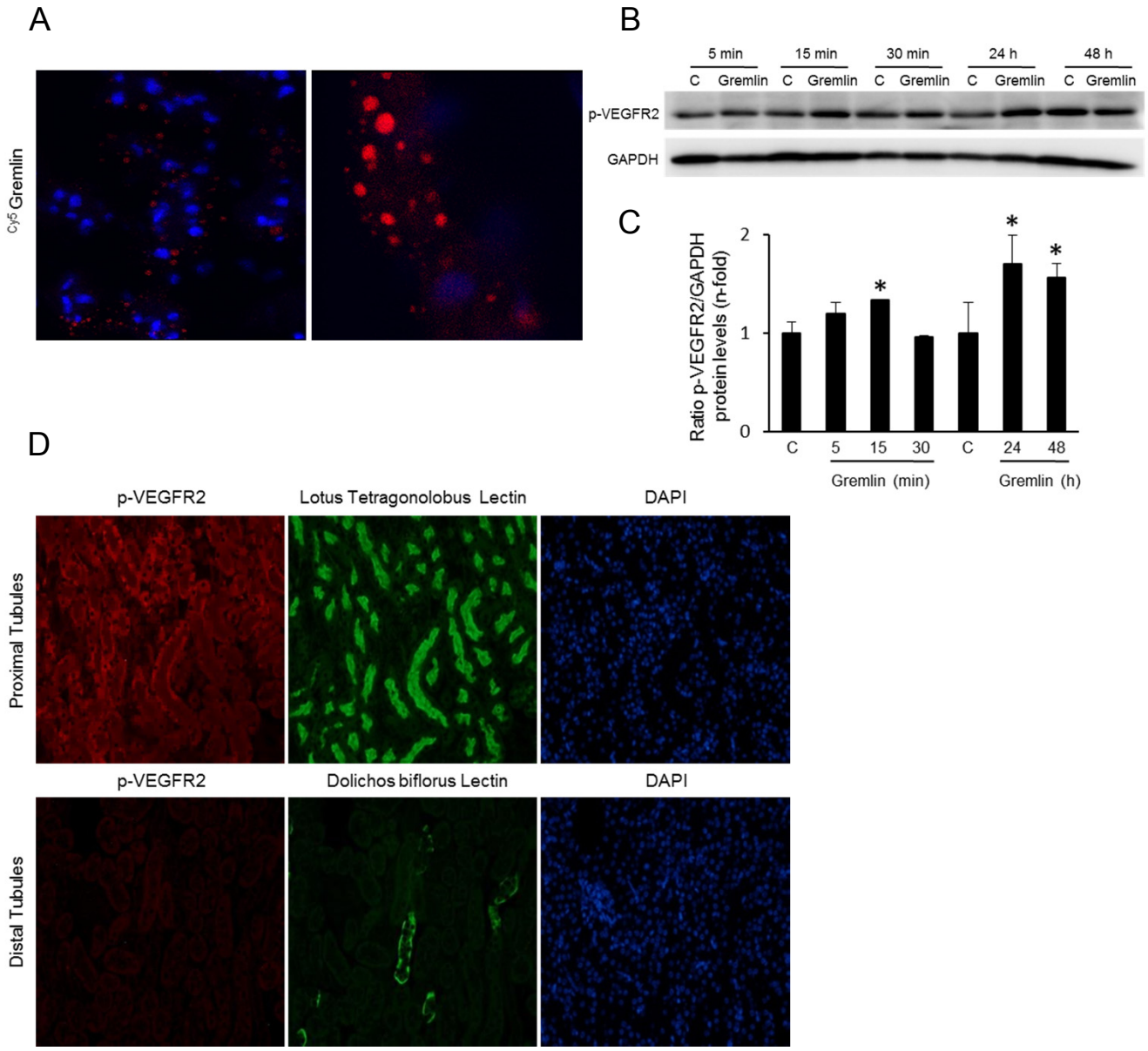

Figure 3. Gremlin binds to tubular epithelial cells *in vivo* in the kidney. C57BL/6 mice were injected with Cy5-Gremlin (50 ng/g of body weight, n=2-4 mice per group) in renal parenchyma, sacrificed at different times (from 5 to 60 min), and frozen OCT-embedded renal samples were used for confocal microscopy. Figure A shows a representative Gremlin-injected mice kidney at 15 min. Red immunostaining was found in tubular epithelial cells, showing Gremlin binding to renal cells. Nuclei are shown in blue (DAPI staining). **Gremlin activates VEGFR2 signaling in the kidney.** Mice were injected with Gremlin (50 ng/g of body weight, n=2-4 mice per group) in renal parenchyma of one kidney, while the contralateral kidney was saline-injected, and used as control. Mice were sacrificed at different times (from 5 min to 48 hours). VEGFR2 phosphorylation was determined in total renal extracts by western blot analysis. Figure **B** shows one representative mice from each group and **(C)** data are expressed as mean±SEM of 6-8 mice per group. *p<0.05 vs contralateral kidney. **(D)** Co-localization of p-VEGFR2 (red) staining with markers of proximal (Lotus Tetragonolobus lectin, green) or distal (Dolichos biflorum agglutinin lectin, green) tubules in Gremlin-injected mice. Proximal tubules are the main sites of p-VEGFR2 staining.

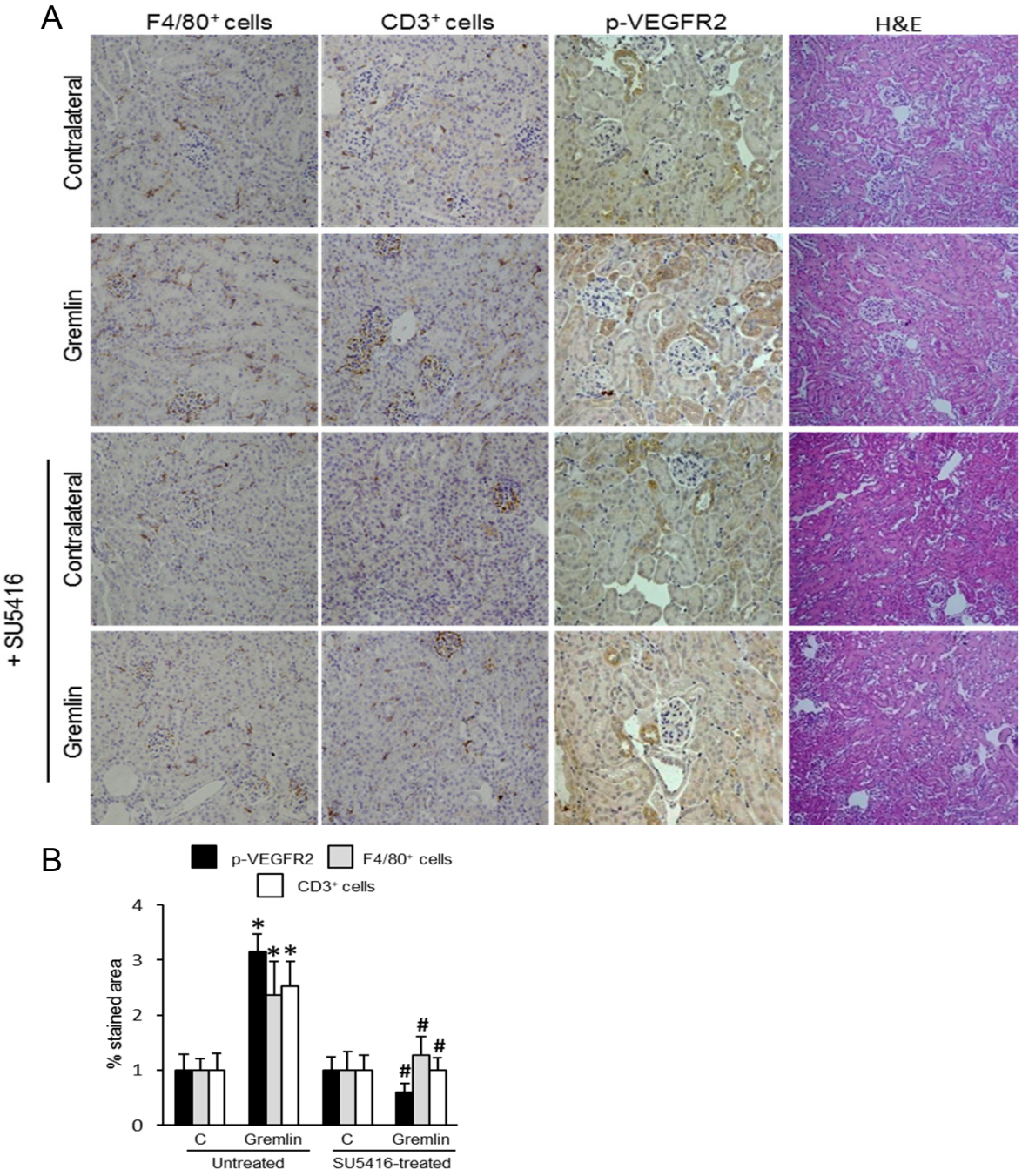

Figure 4. Gremlin causes an inflammatory response in the kidney via VEGFR2 signaling. C57BL/6 mice were injected Gremlin (50 ng/g of body weight) in renal parenchyma of one kidney, while the contralateral kidney was used as control and sacrificed 48 hours later. Some animals were treated with SU5416 (VEGFR2 kinase inhibitor, 0.1 mg/g per day) or its vehicle (control group), starting 24 hours before Gremlin injection. **(A)** In paraffin-embedded kidney sections, immunohistochemistry using anti-F4/80 and anti-CD3 was performed to characterize monocyte/macrophages and T lymphocytes, respectively. Activation of VEGFR2 signaling was evaluated by positive p-VEGFR2 staining. Hematoxylin/eosin (H&E) illustrates morphological changes. Figure A shows representative sections from each group (Magnification 200×), and in **(B)** the quantification of the immunostaining. *p<0.05 vs contralateral untreated kidney; #p<0.05 vs Gremlin-injected kidney.

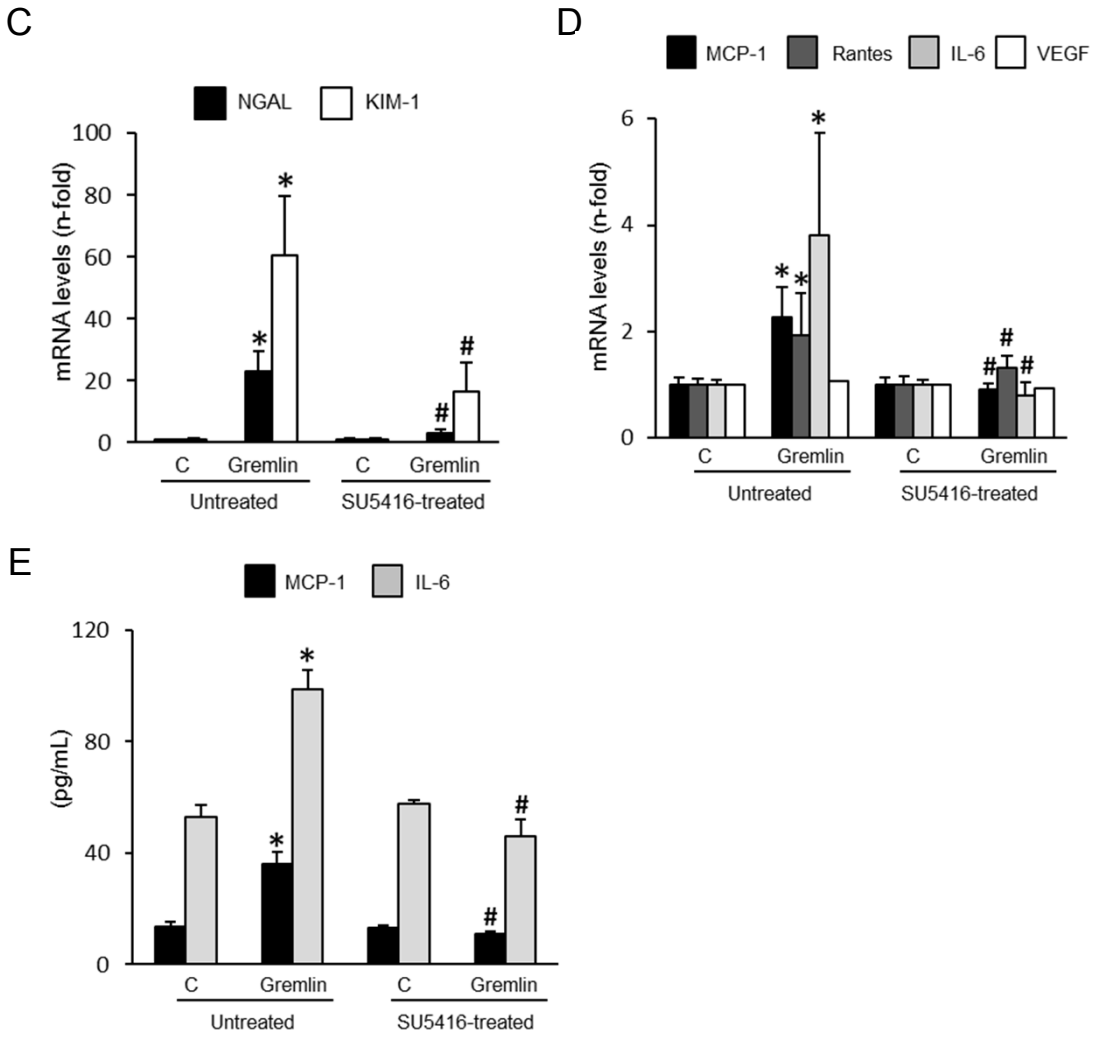

Figure 4. Gremlin via VEGFR2 increases renal expression of biomarkers of renal damage (NGAL and KIM-1) (C) and pro-inflammatory molecules (MCP-1, Rantes and IL-6) (D and E). In frozen samples from total kidneys RNA and proteins were isolated. Gene expression levels were studied by quantitative real-time PCR **(C and D),** and protein levels by ELISA **(E)**. Results are mean±S.E.M. of 6–8 animals per group. Data are normalized vs contralateral untreated kidney (considered as 1). *p<0.05 vs contralateral untreated kidney; #p<0.05 vs Gremlin-injected kidney.

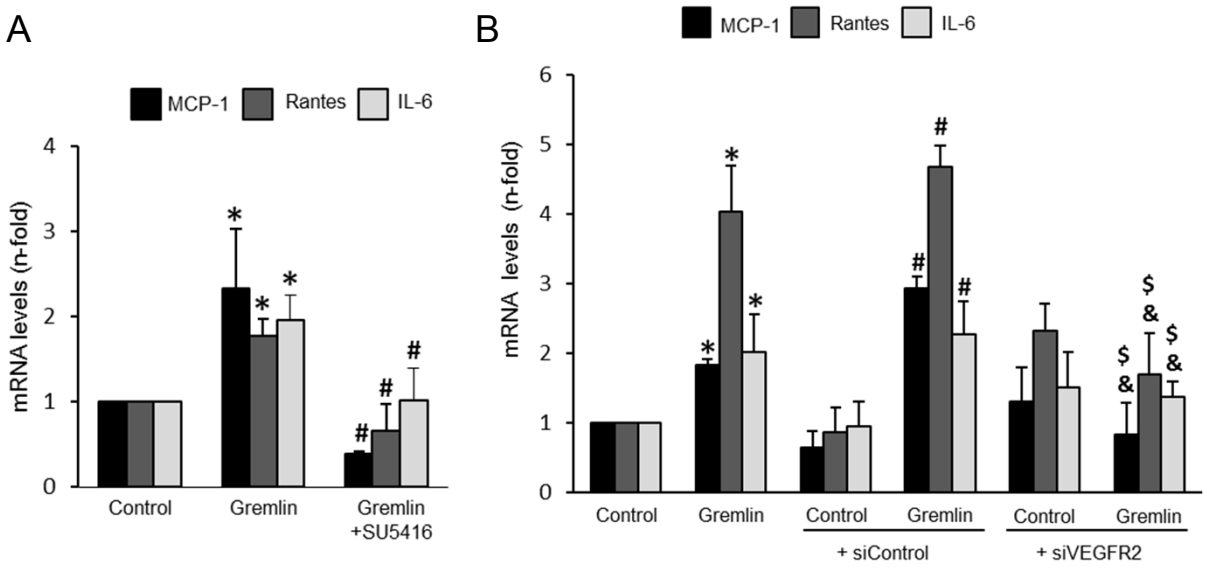

Figure 5. VEGFR2 signaling blockade inhibits Gremlin-mediated pro-inflammatory factors up-regulation in cultured tubular epithelial cells. (A) Cells were pre-incubated for 1 hour with 5 μM SU5416, before stimulation with 10 ng/mL Gremlin for 6 hours. Gene expression levels were studied by quantitative real-time PCR. Data are expressed as mean±SEM of 5 independent experiments. *p<0.05 vs control #p<0.05 vs Gremlin. **(B)** HK2 cells were transfected with a siRNA against VEGFR2 or its corresponding control siRNA. Then, cells were stimulated or not with 10 ng/mL Gremlin for 24 hours. Data are expressed as mean±SEM of 5 independent experiments. *p<0.05 vs control untransfected; #p<0.05 vs untreated scramble siRNA-transfected cells; $p<0.05 vs Gremlin-treated scramble siRNA-transfected cells; &p<0.05 vs Gremlin treated untransfected cells.

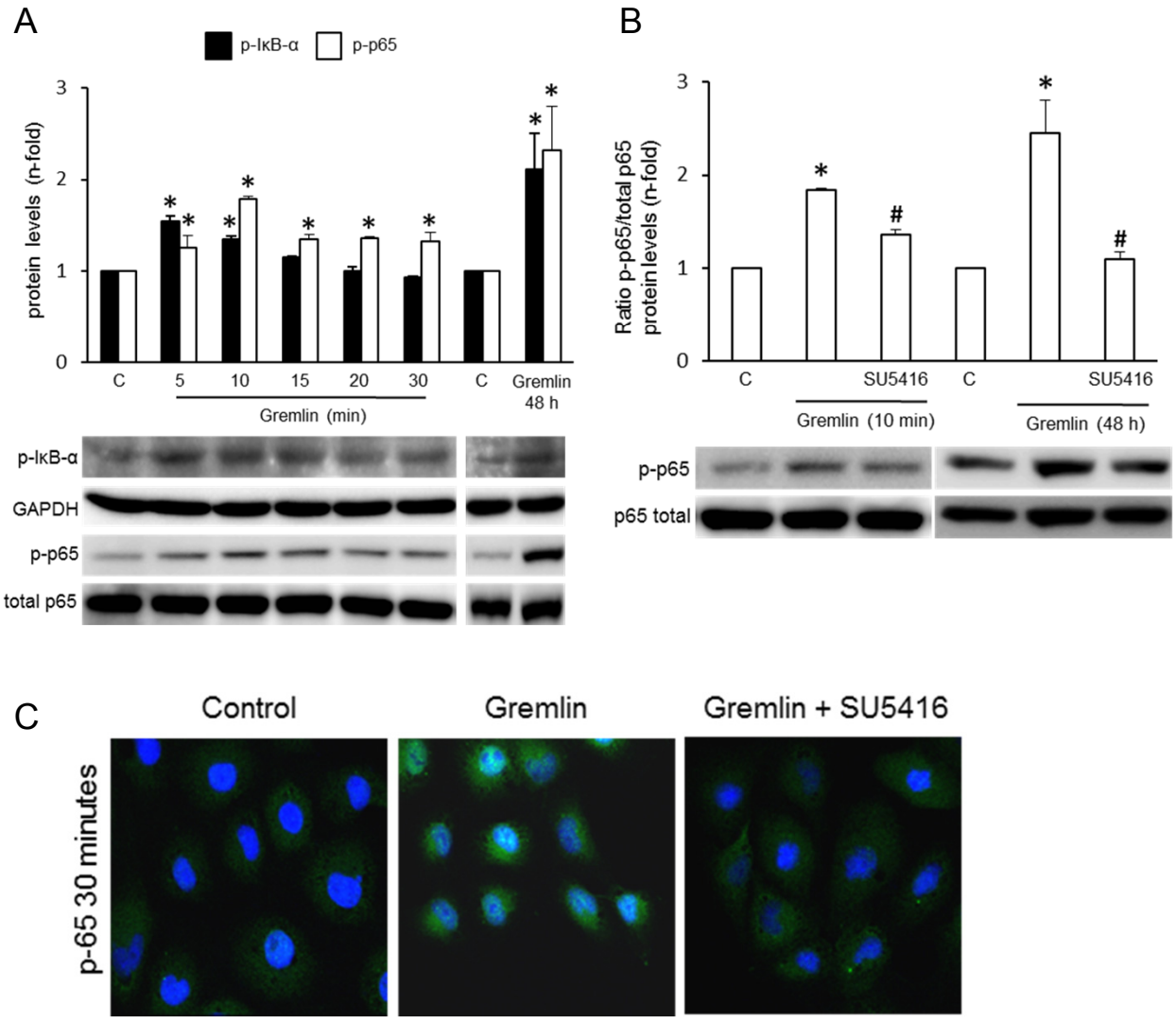

Figure 6. Gremlin, via VEGFR2, activates the NF-κB pathway in cultured tubular epitelial cells. (A) HK2 cells were stimulated with 10 ng/mL Gremlin for different times. Activation of NF-κB was evaluated in total protein extracts by phosphorylation of the IκB-α and NF-κB p65 subunit by Western blotting. GAPDH and total p65 were used as the reference value respectively. **(B)** In some points, cells were pretreated with 5 μM SU5416, before stimulation with 10 ng/mL Gremlin. Data are expressed as mean±SEM of 5 independent experiments. *p<0.05 vs control; #p<0.05 vs Gremlin. **(C)** Gremlin caused nuclear translocation of p65 NF-κB subunit after 30 min of incubation. Indirect immunofluorescence was done using p65 antibody and AlexaFluor488-labeled secondary antibody (green staining). Nuclei were stained with DAPI (blue). Pictures show representative images of 3 independent confocal microscopy experiments.

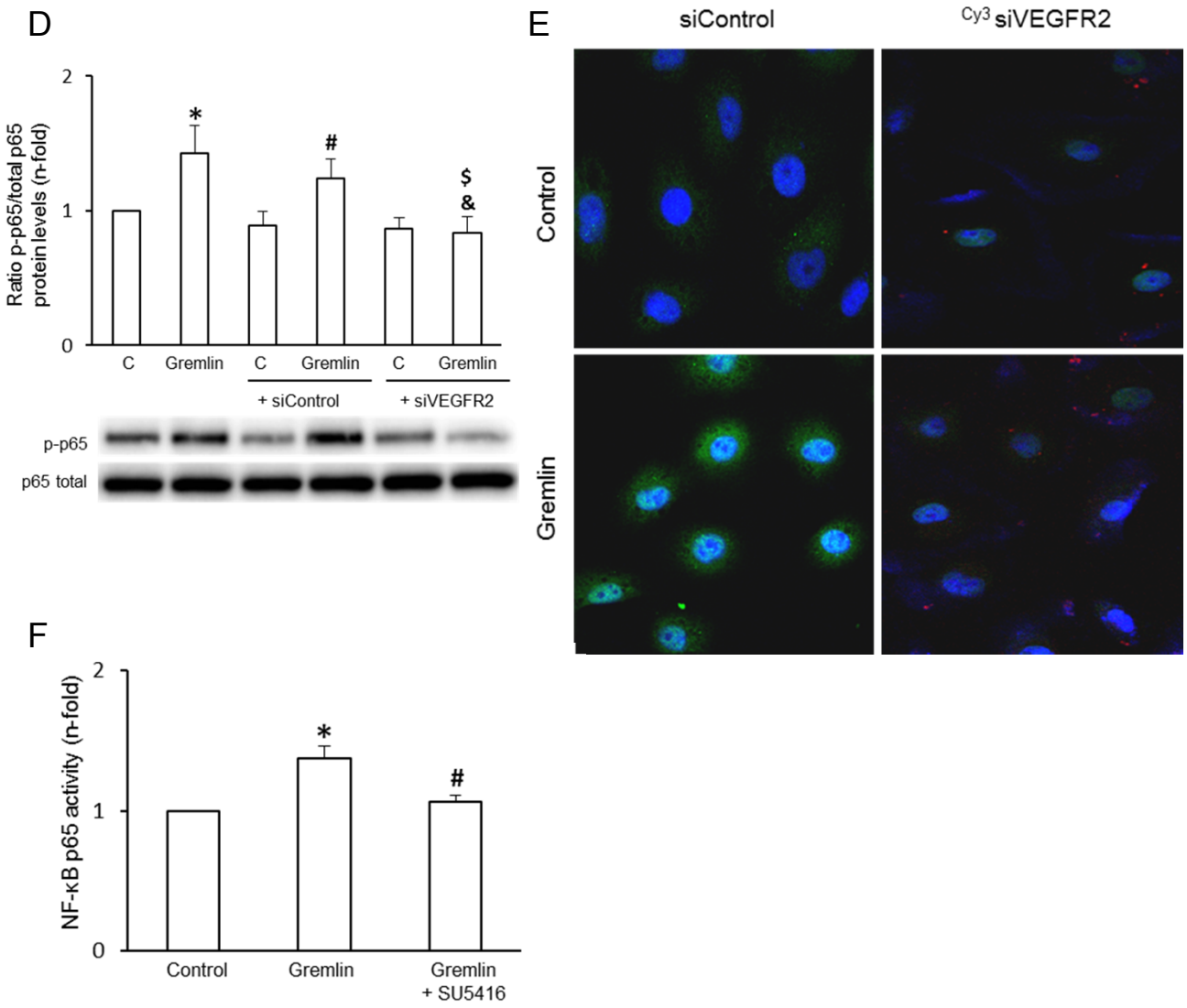

Figure 6. Gremlin, via VEGFR2, activates the NF-κB pathway in cultured tubular epitelial cells. (D) Cells transfected with VEGFR2 siRNA or control siRNA, and then were treated with 10 ng/ml Gremlin for 10 min. Data are expressed as mean±SEM of 5 independent experiments. *p<0.05 vs control untransfected; #p<0.05 vs untreated control siRNA-transfected cells; $p<0.05 vs Gremlin-treated control siRNA-transfected cells; &p<0.05 vs Gremlin-treated untransfected cells. **(E)** Cells were transfected with a siRNA against VEGFR labeled with Cy3 (orange), or control siRNA and then treated or not with 10 ng/ml Gremlin for 30 min. The NF-κB p65 subunit was localized by indirect immunofluorescence (green). **(F)** NF-κB DNA binding activity was measured by ELISA in nuclear proteins using an antibody that only recognizes p65 when NF-κB is activated and bound to the NF-κB consensus site Data are expressed NF-κB p65 activity change compared with control, as mean±SEM of 5 independent experiments. *p<0.05 vs control; #p<0.05 vs Gremlin.

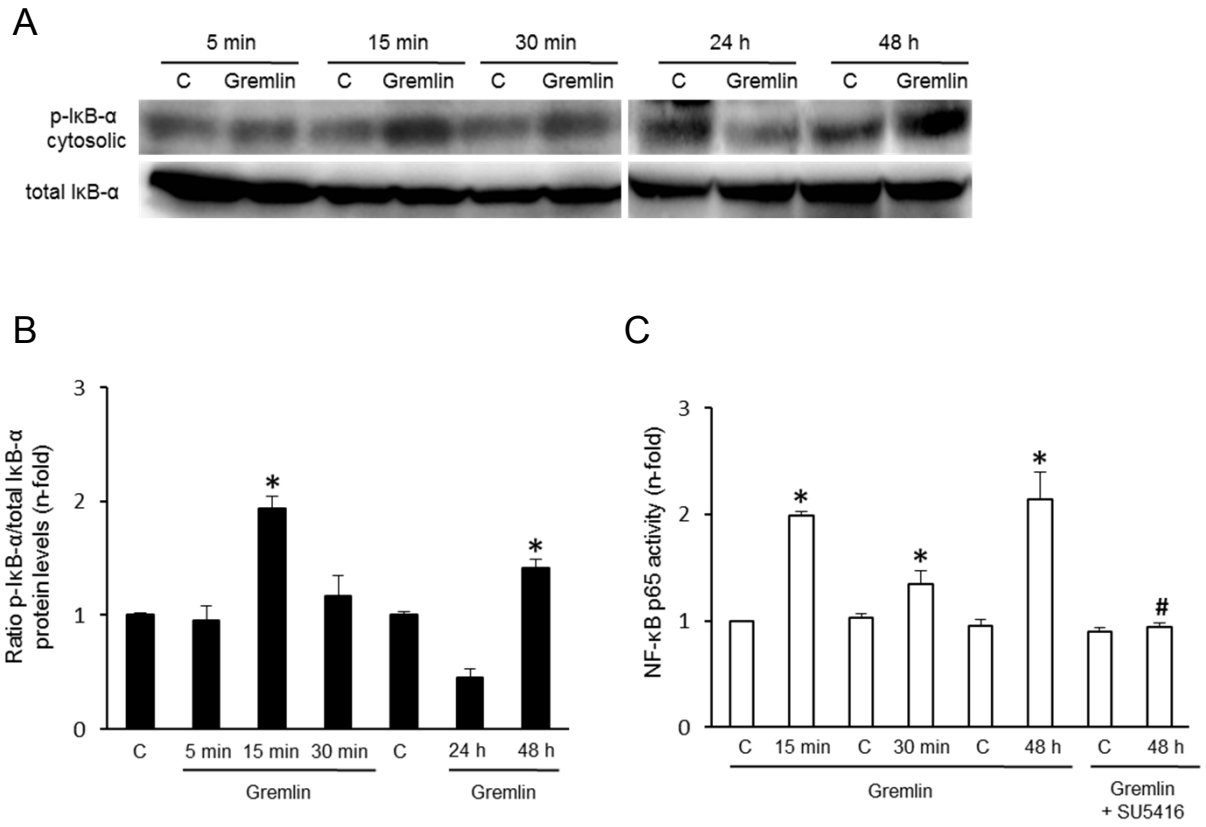

Figure 7. Gremlin activates the NF-κB pathway in the kidney. In Gremlin and contralateral kidneys nuclear and cytosolic proteins were isolated. IκBα phosphorylation (Ser32) and total IκBα was measured in cytosolic renal fractions. Figure **(A)** shows a representative Western blot and in **(B)** phosphorylated IκBα levels after normalization. **(C)** NF-κB p65 DNA binding activity was measured by ELISA in isolated renal nuclear proteins, and absorbance values are normalized vs contralateral kidneys at time 15 min (considered as 1). Data are expressed as mean±SEM of 6-8 animals per group. *p<0.05 vs contralateral kidney; #p<0.05 vs Gremlin-injected.

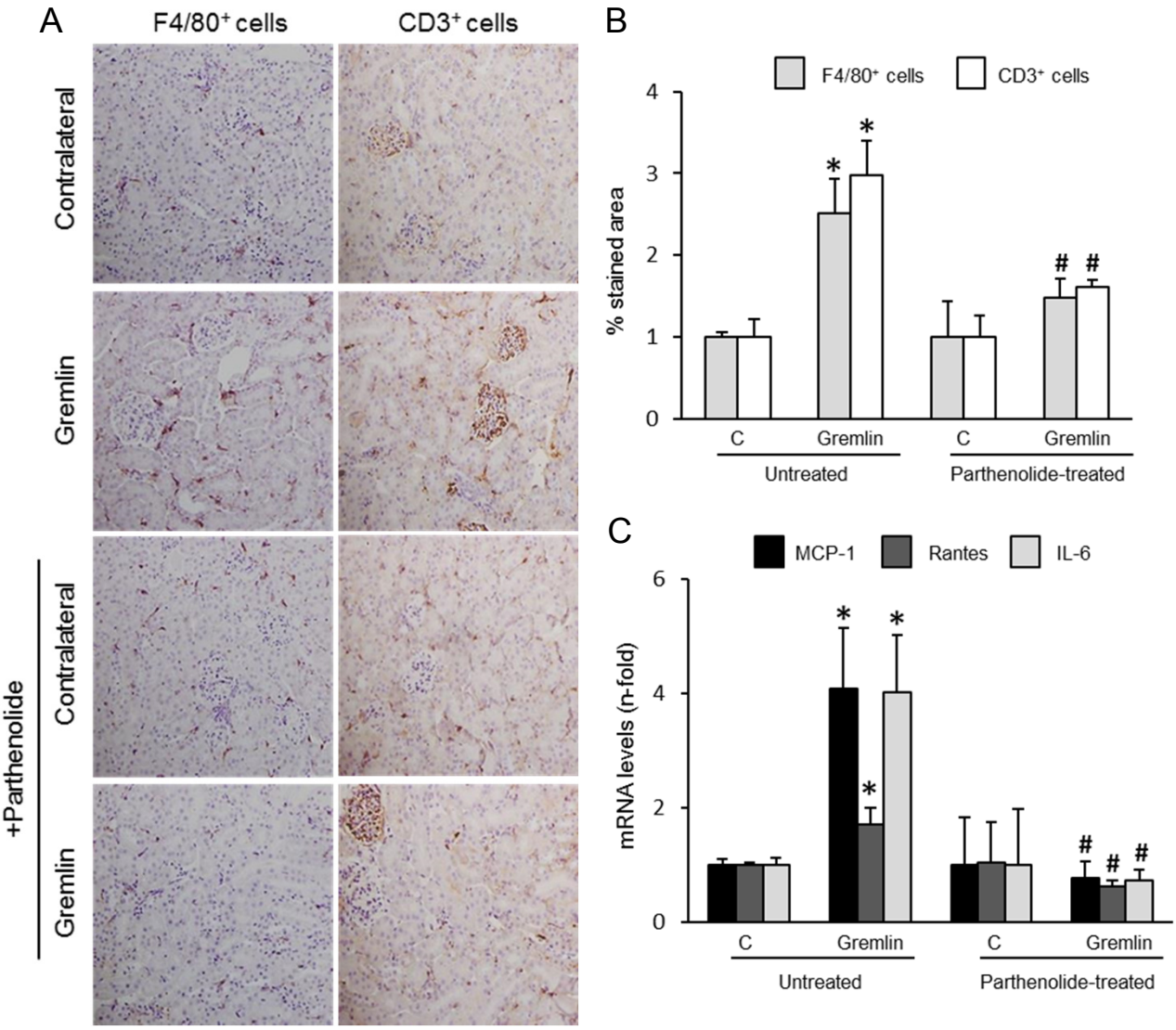

Figure 8. NF-κB inhibition ameliorates Gremlin induced renal injury. Mice were daily treated with the NF-κB inhibitor Parthenolide (3.5 mg/g) or its vehicle (0.01% DMSO, control group), starting 24 hours before Gremlin administration (50 ng/g, renal parenchyma injection in the left kidney). **(A)** Inflammatory cell infiltration was evaluated using anti-F4/80 (macrophages) and anti-CD3 (T lymphocytes) antibodies. **(B)** Immunohistochemistry staining quantification expressed as mean±SEM of 6-8 animals per group. *p<0.05 vs contralateral; #p<0.05 vs Gremlin-injection. **(C)** MCP-1, Rantes and IL-6 renal gene expression are expressed as mean±SEM of 6-8 animals per group analyzed by real-time PCR. *p<0.05 vs contralateral; #p<0.05 vs Gremlin-injection.

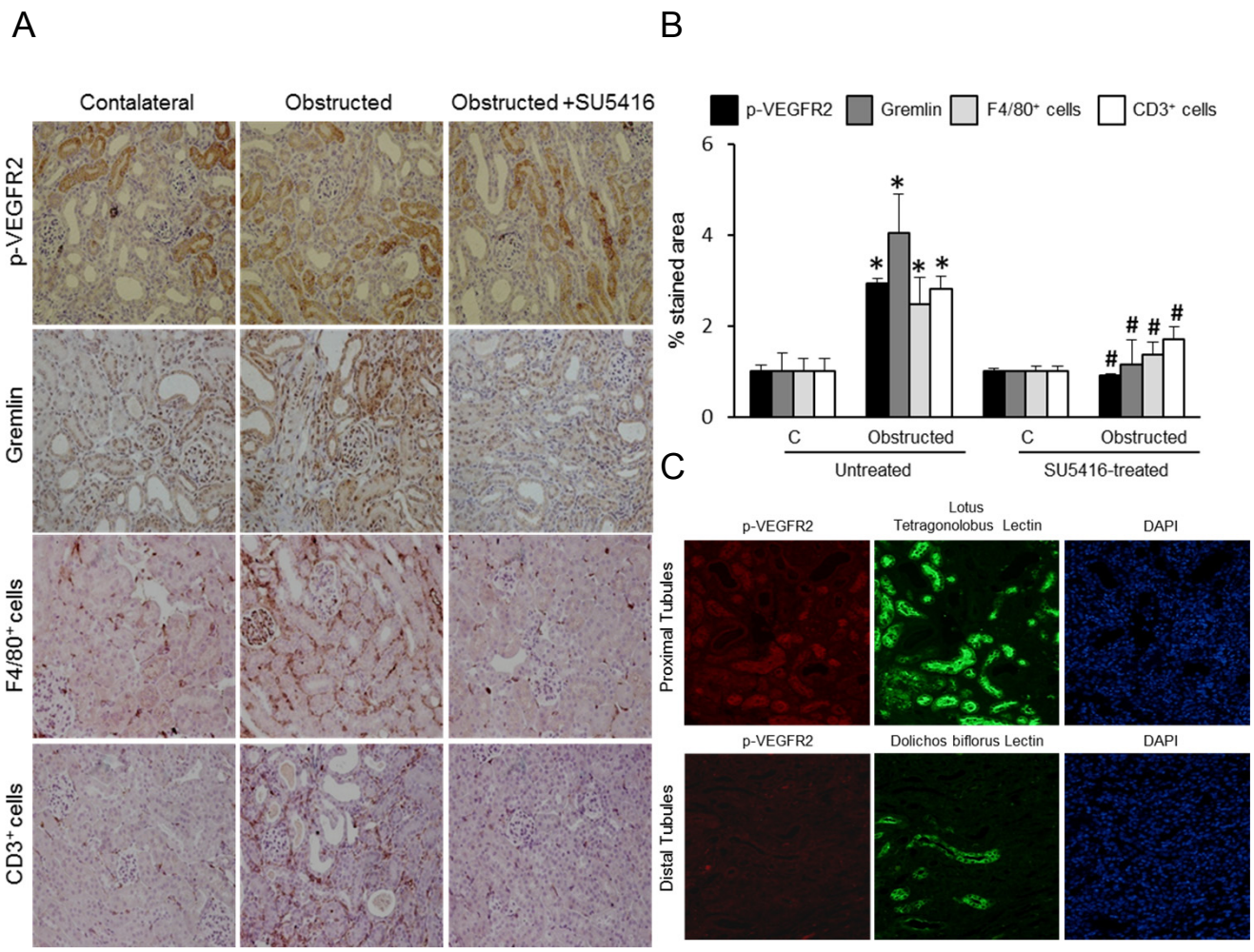

Figure 9. Evaluation of the axis Gremlin/VEGFR2 in the experimental model of Unilateral Ureteral Obstruction (UUO) in mice. Mice were treated with SU5416 (0.1 mg/g per day) or vehicle (0.01% DMSO), starting 24 hours before UUO, and studied after 5 days. In paraffin embedded kidney sections, immunohistochemistry was done using antibodies against VEGFR2, Gremlin, F4/80 and CD3. Figure A shows a representative animal from each group (Magnification 200X) and in **B** their quantification. **(C)** Co-localization of p-VEGFR2 (red) staining with markers of proximal (Lotus Tetragonolobus lectin, green) or distal (Dolichos biflorum agglutinin lectin, green) tubules in obstructed kidneys. Proximal tubules are the main sites of p-VEGFR2 staining.

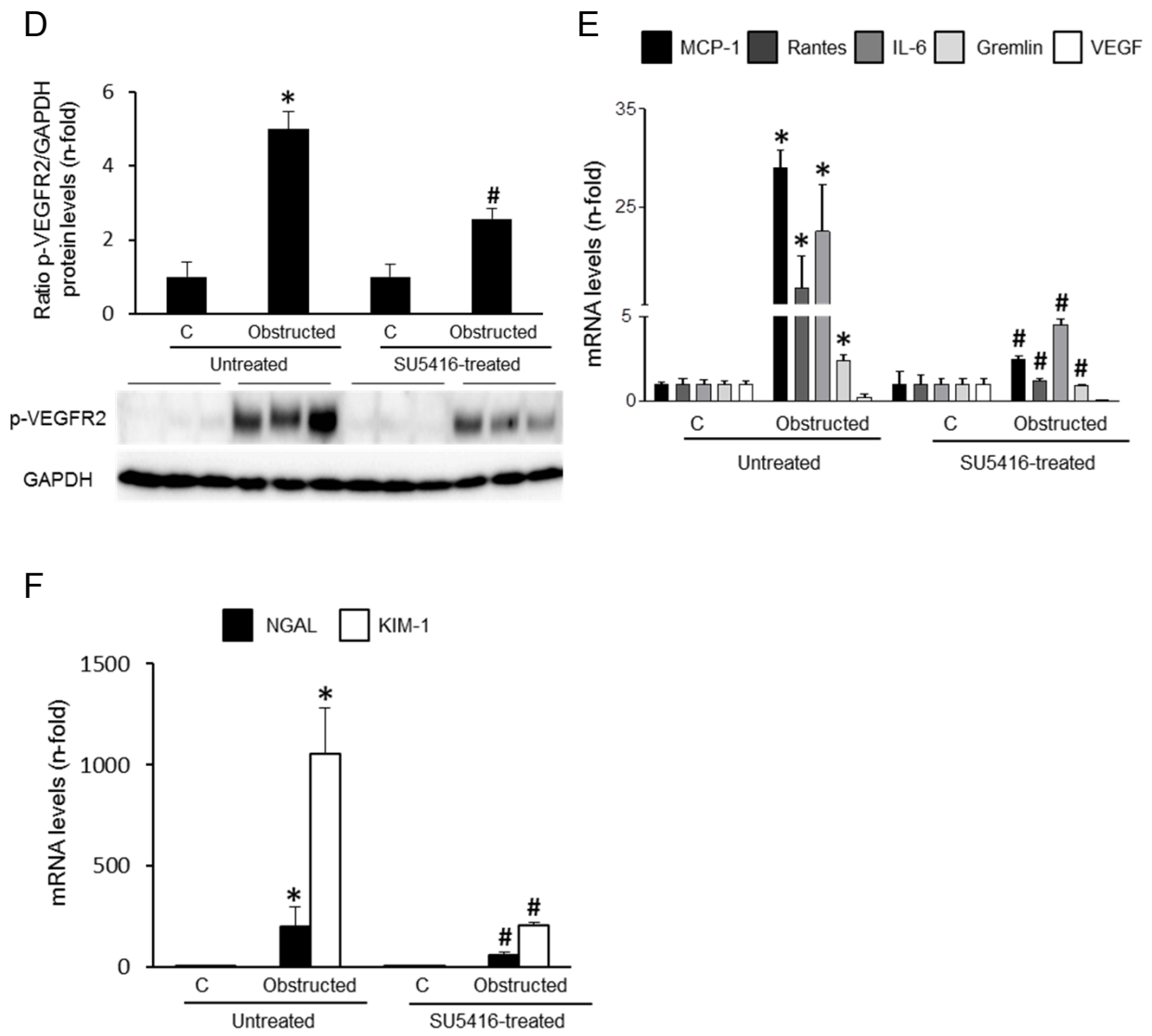

Figure 9. Evaluation of the axis Gremlin/VEGFR2 in the experimental model of Unilateral Ureteral Obstruction (UUO) in mice. **(D)** By western blotting, renal levels of p-VEGFR2 were also assessed (a representative blot is shown). Quantitative real time PCR was performed to determine gene expression levels of **(E)** MCP-1, Rantes, IL-6, Gremlin, VEGF, **(F)** NGAL and KIM-1. Data are expressed as mean±SEM of 6-8 animals per group. *p<0.05 vs contralateral; #p<0.05 vs obstructed kidneys.

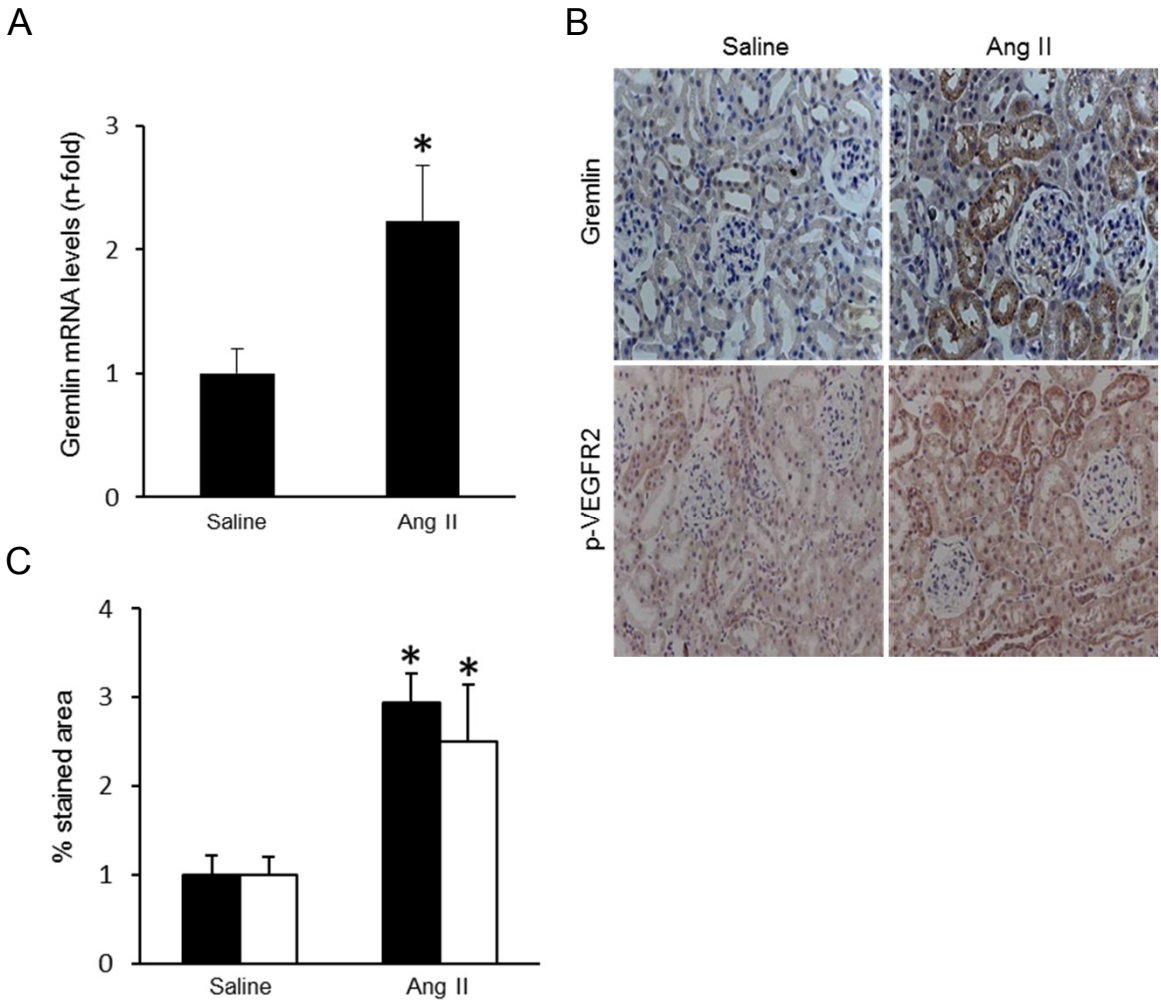

Figure 10. Evaluation of the axis Gremlin/VEGFR2 in AngII-infused rats. Infusion of AngII (100 ng/kg/min) was done in normotensive rats during 2 weeks, saline infusion was used as control. **(A)** Gremlin gene expression was evaluated by real time PCR. Data are expressed as mean±SEM of 8 animals per group. *p<0.05 vs saline infused rats. **(B)** Gremlin and p-VEGFR2 expression were evaluated by immunohistochemistry. **(C)** Quantification of stained area as mean±SEM of 8 animals per group. *p<0.05 vs saline infused rats. Figure shows a representative picture of each group. Original magnification 200x.

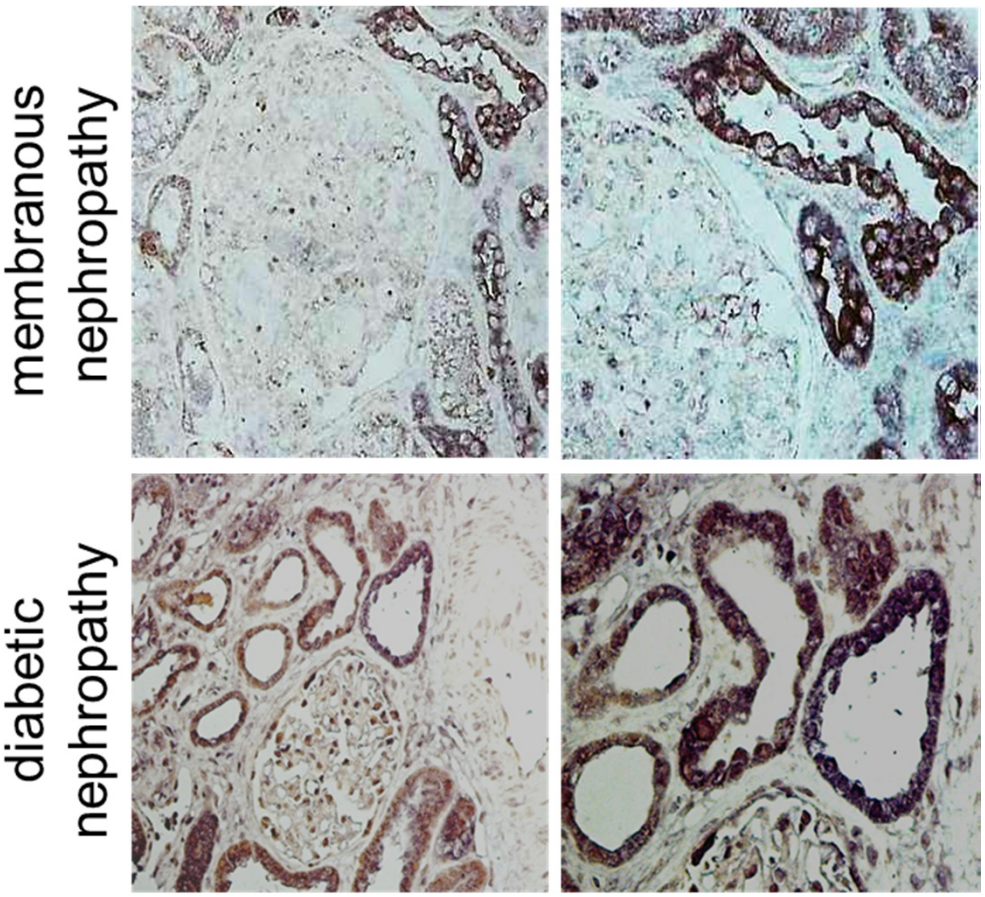

Figure 11. In human progressive nephropathies, Gremlin induction was associated to VEGFR2 activation in the kidney. Immunohistochemistry showing co-localization of Gremlin (brown) and p-VEGFR2 (blue). Studies were done in 8 human biopsies of patients with membranous nephropathy, necrotizing glomerulonephritis and diabetic nephropathy. Magnification 200x and 400x. Figure shows 2 representative cases.

MATERIALS AND METHODS

Cell cultures

Human renal proximal tubular epithelial cells (HK2 cell line, ATCC CRL-2190) were grown in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, 100U/ml penicillin, and 100 μg/ml streptomycin, 5 mg/mL insulin transferrin selenite (ITS) and 36 ng/mL hydorcortisone in 5% CO2 at 37°C. At 60-70% of confluence, cells were growth-arrested in serum-free medium for 24 hours before the experiments. Cells were cultured in six-well plates, serum starved for 24 hours and treated with vehicle, recombinant human Gremlin (10 ng/mL, Peprotech) for 24 or 48 hours in serum-free medium. In some experiments cells were preincubated for 1 hour with VEGFR2 kinase inhibitor SU5416 (5 μM; Vichem, Budapest, Hungary), BMPs (10 μg/ml; Peprotech) or Parthenolide (10^{-6} M; Calbiochem). DMSO, used as solvent, had no effect on cell viability and gene expression (Data not shown). Cell culture reagents were obtained from Lonza.

Live cell confocal microscopy

Cells were imaged using a Leica TCS SP5 confocal microscope. Fluorophore Cy-5-emitted fluorescence was monitored with a 550 ± 2 nm band pass or a 670 nm long pass filter and DAPI was excited using a DIODE laser. The images were captured (1 frame every 1.33 sec) at 400 Hz for a period of 5 min and digitalized using the LIF/LEICA program (LEICA microsystems).

Fluorescence labelling of proteins and siRNAs

Cy-5 fluorophore (1 mM; Amersham) was dissolved in dimethilformamide at 100 mM and used to label Gremlin recombinant protein (dissolved in sterile water, and adjust to pH 8.8, to modify the pH of the protein) following the manufacturer labelling reaction instructions (30 min in darkness and adding 1 ul of lysine 10 mmol/L to stop the reaction). Human KDR/VEGFR2 siRNA was labelled with FAM fluorophore using Ambion's Silencer siRNA Labelling kit following the manufacturer's instructions.

Cell transfection and gene silencing

Gene silencing in cultured cells was performed using either a predesigned siRNA corresponding to the human KDR/VEGFR2 cDNA sequence (s7822; Ambion) or a control non-specific control siRNA (Ambion). Subconfluent HK2 cells were transfected for 24 hours with Lipofectamine™ RNAiMAX reagent (Invitrogen) according to the manufacturer's guidelines. Then, cells were incubated in serum-free medium for 24 h before the experiments. At some points, cells were treated or not with Gremlin for different times.

Experimental models

All animal procedures were performed according to the guidelines of animal research in the European Community and with prior approval by the Ethics Committee of the Health Research of the IIS-Fundación Jiménez Díaz.

The model of intra-renal parenchymal injection of Gremlin was done in 3-month-old female C57BL/6 mice. The model was perfomed under isoflurane-induced anesthesia; mice received parenchymal injection, done as described,[74] of recombinant murine Gremlin (Cy5 labelled or not) at the dose of 50 ng/g of body weight in the left kidney, and analysed at different time points, until 48 hours. The dose chosen of Gremlin was based on the *in vitro* studies. Some animals were daily treated with the VEGFR2 inhibitor SU5416[30] (i.p; 0.1 mg per mice per day, Vichem, Budapest, Hungary) or Parthenolide (i.p; 3,5µg/g of body weight per day), starting 1 day before Gremlin injection (n= 6-8 mice per group), and studied 48 hours later.

The model of unilateral ureteral obstruction (UUO) was done in male C57BL/6 mice. The model was performed under isoflurane induced anesthesia; the left ureter was ligated with silk (4/0) at two locations and cut between ligatures to prevent urinary tract infection (obstructed kidney), as described.[34] Some animals were treated with SU5416 (i.p; 0.1 mg per mice per day) 1 day before UUO and continued for 5 days (n= 8 mice per group).

The model of systemic infusion of Ang II was done in 3-month old male Normotensive Wistar-Kyoto rats (WKY, Criffa, Barcelona, Spain). Ang II (Biochem) dissolved in saline was infused at the dose of 100 ng/kg/min by subcutaneous osmotic minipumps (Alza Corp) for 2 weeks (n= 8 rats per group). A control group of saline-infused rats of the same age was also studied (n= 8 rats per group).

At the time of sacrifice, animals were anesthetized with 5 mg/kg xylazine (Rompun, Bayer AG) and 35 mg/kg ketamine (Ketolar, Pfizer) and the kidneys perfused *in situ* with cold saline before removal. A piece of the kidney (2/3) was fixed, embedded in paraffin, and used for immunohistochemistry, and the rest was snap-frozen in liquid nitrogen for renal cortex RNA and protein studies. In both models, studies were done comparing both kidneys (contralateral and obstructed) in each mouse. In addition, a control group of sham-operated mice was also done, showing the same results than contralateral kidneys (data not shown).

Histology and imunohistochemistry

Paraffin-embedded kidney sections were stained using standard histology procedures. Immunostaining was carried out in 5 µm thick tissue sections. Antigen retrieval was performed using the PTlink system (Dako) with sodium citrate buffer (10 mM) adjusted to pH 6–9, depending on the immunohistochemical marker. Endogenous peroxidase was blocked. Tissue sections were incubated for

1 h at room temperature with 4% in PBS to eliminate non-specific protein binding sites. Primary antibodies were incubated overnight at 4ºC. Specific biotinylated secondary antibodies (Amersham Biosciences) were used, followed by streptavidin–horseradish peroxidase conjugate, and 3,3-diaminobenzidine as a chromogen, then sections were counterstained with Carazzi's haematoxylin. The specificity was checked by omission of primary antibodies. Quantification was made by determining the total number of positive cells in ten randomly chosen fields (×200 magnification) using Image-Pro Plus software. Data are expressed as the positive-stained area compared with the total analysed area. The following primary antibodies were employed: phosphorylated-VEGFR2 (p-VEGFR2 (Tyr951) [1:100], Santa Cruz); Gremlin ([1:100], Abgent); CD3 (ready to use, Dako) and F4/80 ([1:50], Serotec).

Immunofluorescence was used to discriminate between proximal tubular and distal tubular expression of p-VEGFR2. For these studies, renal sections were stained with anti-p-VEGFR2 [1:100] and secondary AlexaFluor®633 conjugated anti-rabbit ([1:300], Invitrogen) followed by incubation with the proximal tubule marker FITC-conjugated Lotus Tetragonolobus Lectin ([1:33]; Sigma) or the collecting tubule marker FITC-conjugated Dolichos biflorus lectin ([1:33]; Sigma), as described.[75]

Protein studies

Total protein samples for frozen renal tissue were isolated in lysis buffer [(in mM): 50 Tris/HCl (pH 7.4), 150 NaCl, 2 EDTA, 2 EGTA)] with 0.2% Triton X-100, 0.3% IGEPAL and Complete™ protease inhibitor cocktail (Roche), and used for western blot and ELISA experiments (for cytokines evaluation). In other experiments, nuclear and cytoplasmic fractions were separated from renal tissues using the NE-PER Reagent (Pierce) following the manufacturer's instructions. Cytoplasmic fractions were used in Western blot analysis and nuclear proteins were used to determiner p65 DNA binding activity by ELISA. MCP-1 (monocyte chemoattractant protein-1) and IL-6 (interleukin-6) total renal protein levels were evaluated by ELISA (BD Bioscience). For Western blot analysis, cytosolic fractions and total protein levels were quantified using a BCA protein assay kit (Pierce) with BSA as the standard. Proteins (20-100 µg/lane) were separated on 8-12% polyacrylamide-SDS gels under reducing conditions. Samples were then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), blocked in PBS containing 0.1% Tween 20 and 5% dry non-fat milk for 1 hour at room temperature, and incubated in the same buffer with different primary antibodies overnight at 4 ºC. Subsequently were incubated with the appropriate HRP (horseradish peroxidase)-conjugated secondary antibody (Amersham Biosciences), and developed by ECL kit (Amersham Biosciences). The quality of proteins and efficacy of protein transfer were evaluated by Red Ponceau staining (results not shown). The loading controls used were: GAPDH (for total proteins). Autoradiographs were scanned using the GS-800 Calibrated Densitometer (Quantity One; Bio-Rad Laboratories). The following primary antibodies were employed: phosphorylated VEGFR2

(Tyr996) ([1/500], Santa Cruz); VEGFR2 ([1/500], Santa Cruz); p-p65 (Ser536) ([1/500], Cell Signaling); p65 ([1/500], Santa Cruz); p-IκB-α (Ser32)([1/500], Santa Cruz); IκB-α ([1/500], Santa Cruz) and GAPDH ([1/5000]; Chemicon International).

Chemical Cross-linking and coimmunoprecipitation assays

For these experiments, cells were incubated with Gremlin for 5 min, and then chemical cross-linking was carried out as described previously, with minor modifications.[76] Briefly, the cells were washed three times with cold PBS [(in mM): (137 NaCl, 0.67 KCl, 8 Na_2HPO_4, 1.4 KH_2PO_4)] and incubated for 30 min at 4°C with 1 mM DTSSP (3,3´-dithiobis [sulfosuccinimidylpropionate]) (Pierce Chemical Co) in PBS, followed by washing three times with Tris-buffered saline (TBS) [(in mM): (20 Tris-HCl, 100 NaCl, pH 7.5) before use in the following immunoprecipitation and immunoblot experiments. Cells were lysed in 500 μl lysis buffer [(in mM): 50 Tris-HCl pH 8, 150 NaCl, 1 phenylmethylsulphonylfluoride, 1% NP-40/IGEPAL, and a phosphatase-inhibitor cocktail (Set II, Calbiochem)], scraped off the dish and incubated 1 h to 4ºC with shaking. Cell lysates were precleared by incubating with 10 μl of protein A-agarose bead slurries (0.5 ml agarose/2 ml phosphate-buffered saline) for 30 min at 4°C, and then centrifuged three times for 5 minutes at 2500 rpm, to wash supernatants. Precleared lysates were incubated with a rabbit polyclonal anti-VEGFR2 agarose conjugated antibody (Santa Cruz, 20 μl) overnight at 4°C. The agarose beads were collected by centrifugation, washed three times with lysis buffer, resuspended in 2x Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE. Then, western blot was done using an anti-Gremlin antibody. For loading control, an anti-VEGFR2 antibody was employed ([1:1000]; Santa Cruz).

Immunocytochemistry staining of the NF-κB p65 subunit

Immunocytochemistry studies were performed in cells seeded in 24-well plates on glass coverslips. Human KDR/VEGFR2 siRNA was labelled with Cy3 (indocarbocyanine) using Ambion's Silencer siRNA Labelling kit following the manufacturer's instructions. The transfection protocol was performed as described above. Then, cells were treated or not with Gremlin for 45 minutes. After the experiments, cells were fixed in paraformaldehyde 4% and permeabilized with 0.2% Triton-X100. After blocking with 3% BSA for 1 hour, they were incubated with an anti-p65 ([1/500]; Santa Cruz) antibody overnight at 4ºC, followed by an AlexaFluor® 488 conjugated antibody ([1/300]; Invitrogen). Nuclei were stained with 1 μg/ml 4′,6-Diamidino-2-phenyindole, dilactate (DAPI) (Sigma-Aldrich), as control of equal cell density. Absence of primary antibody was used as negative control. Samples were mounted in Mowiol 40-88 (Sigma-Aldrich) and examined by a Leica TCS SP5 confocal microscope.

Gene expression studies

Total RNA from cells or renal tissue (previously pulverized in metallic chamber) was isolated with TriPure reagent (Roche). cDNA was synthesized by High capacity cDNA Archive Kit (Applied) using 2 µg of total RNA primed with random hexamer primers following the manufacturer's instructions. Next, quantitative gene expression analysis was performed by real-time PCR on an AB7500 fast real-time PCR system (Applied Biosystems) using fluorogenic TaqMan MGB probes and primers designed by Assay-on-Demand™ gene expression products. Mouse assays IDs were: *Mcp-1*, Mm00441242_m1; *Rantes* (regulated upon activation, normal T-cell expressed and secreted), Mm01302428_m1; *Il-6*, Mm00446190_m1; *Ngal* (neutrophil gelatinase-associated lipocalin), Mm01324470_m1; *Kim-1* [kidney injury molecule 1, also known as Havcr1 (hepatitis A virus cellular receptor 1)], Mm00506686_m1; *VEGFA* (vascular endothelial growth factor A), Mm01281449_m1; *Fibronectin*, Mm01256734_m1 and *Gremlin*, Mm00483888_s1. Human assay IDs were: *MCP-1*, Hs00234140_m1; *RANTES,* Hs00174575_m1 and *IL-6*, Hs00174131_m1. Data were normalized to 18s eukaryotic ribosomal RNA expression, assay ID: 4210893E (Vic). The mRNA copy numbers were calculated for each sample by the instrument software using Ct value ("arithmetic fit point analysis for the lightcycler"). After normalization to internal controls, the gene fold expression was calculated from the difference between the gene expression of the experimental condition and the untreated control (unstimulated cells or control mice).

Statistical analysis

Results throughout the text are expressed as n-fold increase over control as mean±SEM. Differences between groups were assessed by Mann-Whitney test. Statistical significance was assumed when a null hypothesis could be rejected at $p<0.05$. Statistical analysis was performed using the SPSS statistical software, version 16.0, Chicago, IL.

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2. Gremlin es un mediador profibrótico del Factor de Crecimiento Transformante-β en células renales en cultivo

Las ERC se caracterizan por acumulación de componentes de la MEC en el túbulo-intersticio.[86] Muchos estudios consideran a los fibroblastos como las principales células productoras de MEC y en gran parte son generados por el proceso de TEM.[85,164,226,228,274] TGF-β1 es uno de los principales factores involucrados en fibrosis renal y es considerado el principal inductor de TEM en el riñón.[17,219,268,269] En estudios de biopsias de pacientes con ERC, se ha demostrado la correlación de la expresión de Gremlin con la de TGF-β1 principalmente en áreas de fibrosis túbulo-intersticial[28,46,147] pero su potencial rol como mediador de TGF-β1 no ha sido demostrado. La investigación de los mecanismos implicados en la fibrosis renal y la identificación de nuevos mediadores con potencial aplicación terapéutica tiene especial relevancia en la enfermedad renal crónica. El primer objetivo de este trabajo fue evaluar si Gremlin podría modular directamente procesos fibróticos en células renales en cultivo. Los resultados obtenidos muestran que en fibroblastos renales murinos (TFBs) en cultivo, Gremlin activa factores pro-fibróticos (TGF-β, CTGF y PAI-1), así como las principales proteínas de la MEC (Fibronectina y Colágeno tipo I). Asimismo, en células túbulo-epiteliales (HK2) en cultivo, Gremlin produce cambios fenotípicos asociados a TEM, siendo capaz de activar la producción de los marcadores mesenquimáticos Vimentina, α-SMA y Fsp-1 y modular la actividad de la MMP-9, además de inhibir la expresión de los marcadores epiteliales E-cadherina y pan-Citoqueratina. Nuestro segundo objetivo fue evaluar si Gremlin es mediador de las acciones de TGF-β, para ello en células estimuladas con TGF-β se realizó silenciamiento génico de Gremlin endógeno mediante la utilización de un siRNA. El bloqueo de Gremlin en TFBs provoca la inhibición de la producción de factores pro-fibróticos tales como TGF-β, CTGF y PAI-1 y de las proteínas de MEC Fibronectina y Colágeno tipo I, y en células HK2 además inhibe cambios asociados a TEM como Vimentina y actividad de MMP-9. Todos estos datos sugieren que Gremlin podría ser un importante modulador de fibrosis renal y una potencial nueva diana terapeútica anti-fibrótica para las enfermedades renales crónicas.

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Gremlin Is a Downstream Profibrotic Mediator of Transforming Growth Factor-Beta in Cultured Renal Cells

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Key Words
Gremlin · Transforming growth factor-beta · Renal fibrosis · Epithelial mesenchymal transition

Abstract
Background/Aims: Chronic kidney disease is characterized by accumulation of extracellular matrix in the tubulointerstitial area. Fibroblasts are the main matrix-producing cells. One source of activated fibroblasts is the epithelial mesenchymal transition (EMT). In cultured tubular epithelial cells, transforming growth factor-β (TGF-β_1) induced Gremlin production associated with EMT phenotypic changes, and therefore Gremlin has been proposed as a downstream TGF-β_1 mediator. Gremlin is a developmental gene upregulated in chronic kidney diseases associated with matrix accumulation, but its direct role in the modulation of renal fibrosis and its relation with TGF-β has not been investigated. ***Methods:*** Murine renal fibroblasts and human tubular epithelial cells were studied. Renal fibrosis was determined by evaluation of key profibrotic factors, extracellular matrix proteins (ECM) and EMT markers by Western blot/confocal microscopy or real-time PCR. Endogenous Gremlin was targeted with small interfering RNA. ***Results:*** In murine fibroblasts, stimulation with recombinant Gremlin upregulated profibrotic genes, such as TGF-β_1, and augmented the production of ECM proteins, including type I collagen. The blockade of endogenous Gremlin with small interfering RNA inhibited TGF-β_1-induced ECM upregulation. In tubular epithelial cells Gremlin also increased profibrotic genes and caused EMT changes: phenotypic modulation to myofibroblast-like morphology, loss of epithelial markers and induction of mesenchymal markers. Moreover, Gremlin gene silencing inhibited TGF-β_1-induced EMT changes. ***Conclusions:*** Gremlin directly activates profibrotic events in cultured renal fibroblasts and tubular epithelial cells. Moreover, endogenous Gremlin blockade inhibited TGF-β-mediated matrix production and EMT, suggesting that Gremlin could be a novel therapeutic target for renal fibrosis.

Introduction

Irrespective of the underlying cause, chronic kidney disease is linked with the development of tubulointerstitial fibrosis, characterized by excessive deposition of extracellular matrix proteins (ECM) [1]. The key cellular player in fibrosis is the activated fibroblast. Fibrogenic fibroblasts can be originated either by proliferation of res-

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ident fibroblasts, or derive from multiple parental lineages, including tubular epithelial cells, endothelial cells, bone marrow endosteal cells, and, as described more recently, from pericytes [1, 2]. Although many studies have investigated the mechanisms of chronic progressive renal fibrosis, there is not an antifibrotic therapy in clinical human use.

Gremlin is a bone morphogenetic protein (BMP) antagonist that belongs to the cysteine knot protein superfamily that includes transforming growth factor-β (TGF-β) proteins. Gremlin has been highly conserved during evolution and has an important role in limb development and neural crest cell differentiation [3–5]. However, in the adult kidney its role in normal and pathological conditions is still unclear. Several authors have suggested that Gremlin could be considered as a mediator of renal injury in diabetic nephropathy. In human biopsies of diabetic nephropathy, we have observed the association of Gremlin expression with tubulointerstitial fibrosis [6]. Experimental studies in the streptozotocin-induced model of type 1 diabetes in knockout mice heterozygous for the grem1 gene showed that several fibrotic-related proteins, such as fibronectin and connective tissue growth factor (CCN-2), were attenuated in grem1(+/–) mice compared with wild-type controls [7]. Recently, in a mouse model of diabetic nephropathy, Gremlin inhibition ameliorated proteinuria and renal type IV collagen accumulation and inhibited mesangial cell proliferation and apoptosis [8]. In contrast, adenovirus-mediated overexpression of Gremlin in rat lungs resulted in the activation of alveolar epithelial cells with proliferation and apoptosis, as well as partly reversible lung fibrosis [9]. However, there are no studies of the direct effect of Gremlin on fibrogenic events in renal cells.

TGF-β_1 is a key factor in renal fibrosis. In tubular epithelial cells Gremlin induction by TGF-β was associated to changes in epithelial-mesenchymal transition (EMT) markers [6], but its potential role as a downstream mediator of TGF-β has not been demonstrated. The aim of the present work was to evaluate whether Gremlin could modulate fibrotic processes in renal cells and its relation with TGF-β-mediated fibrosis.

Materials and Methods

Cell Culture

Murine renal cortical fibroblasts (TFBs, obtained from Dr. Eric Neilson, Vanderbilt University [10]), and human renal proximal tubular epithelial cells (HK2 cell line, ATCC;CRL-2190) were grown as described [11]. HK2 cells were stimulated with human recombinant Gremlin-1 and TFBs with murine Gremlin (R&D), and both cells with human recombinant TGF-β_1 (Peprotech). Cell proliferation was evaluated by MTT kit (Sigma-Aldrich).

Table 1. Gene expression assays used for real-time PCR experiments

Gene	Assay
TGF-β murine	Mm01178819_m1
TGF-β human	Hs00171951_m1
CCN-2 murine	Mm00515790_g1
CCN-2 human	Hs00170014_m1
PAI-1 murine	Mm00435860_m1
PAI-1 human	Hs00167155_m1
Fibronectin	Mm01256734_m1
Collagen type I	Mm00483888_m1
Vimentin	Hs500185584_m1
E-cadherin	Hs500170423_m1
18S	4210893E (Vic)

Gene and Protein Studies

Total RNA was isolated with Trizol (Invitrogen), and multiplex real time PCR was performed using Applied Biosystems expression assays (table 1). Results were expressed in copy numbers, relative to unstimulated cells after normalization against 18S, as described [11]. Gene silencing was performed using a predesigned siRNA corresponding to the human Grem1 cDNA sequence (NM_013372.5) (Ambion), according to the manufacturer's instructions.

Conditioned media (30 μl) or protein extracts (10 μg/lane quantified by the BCA method) were evaluated by Western blot. Metalloprotease activity was measured by zymography. Immunocytochemistry studies were performed using primary antibodies followed by AlexaFluor-488 conjugated antibody (Invitrogen: 1/300) and examined by a Leica DM-IRB confocal microscope. As control of the immunocytochemistry, samples were incubated in the absence of primary antibody (not shown). To normalize ECM data by cell number, nuclei were stained (1 μg/ml DAPI) and quantified using the Image-Pro plus (Media Cybernetic, Inc.). The data are expressed as an arbitrary quantification of the integrated optical density, calculated as average of density of fluorescence per area. These data are shown as n-fold of increase vs. control of the representative experiment shown in the corresponding figure. The antibodies employed were fibroblast-specific protein 1 (DAKO; IF 1/200), vimentin (BD Pharmingen; IF 1/200, WB 1/10,000), E-cadherin (R&D; IF 1/200), Gremlin (Amgen; IF 1/200), α-SMA (Sigma-Aldrich; IF 1/100, WB 1/1,000), pan-cytokeratin (Sigma-Aldrich; IF 1/200, WB 1/2,500), fibronectin and type I collagen (Millipore; WB 1/1,000, IF 1/300), GAPDH (Santa Cruz; WB 1/5,000).

Statistical Analysis

Results are expressed as n-fold increase over control as mean ± SEM. Differences between groups were assessed by Mann-Whitney test. $p < 0.05$ was considered significant. Statistical analysis was conducted using the SPSS statistical software (version 11.0).

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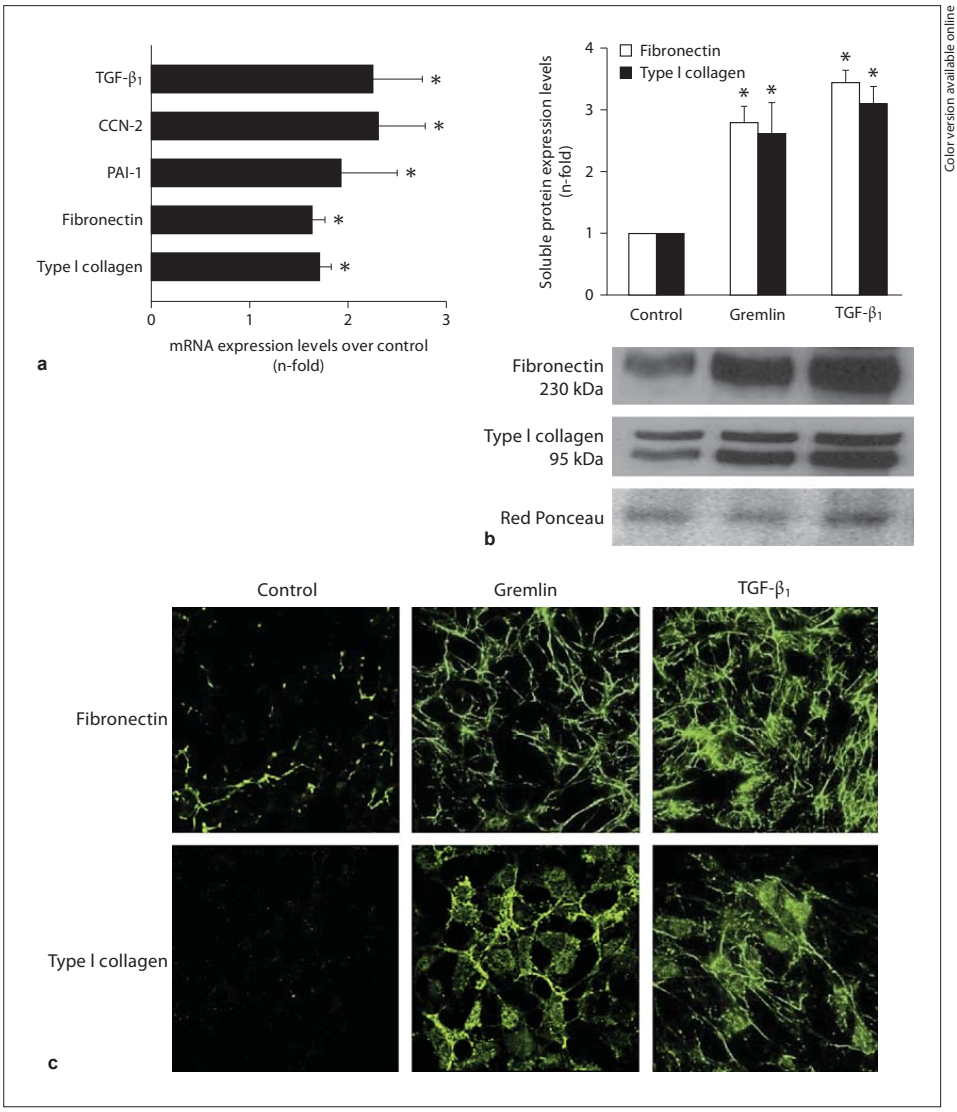

Fig. 1. Gremlin upregulates gene expression of profibrotic and ECM-related factors in murine renal fibroblasts. Cells were stimulated with Gremlin (50 ng/ml murine recombinant protein) for 24 h in serum-free medium and compared to unstimulated cells (control). **a** Gene expression of TGF-β_1, CCN-2, PAI-1, fibronectin and type I collagen was evaluated by real-time PCR. Data are expressed as n-fold over control (considered as 1), as the mean ± SEM of 4 independent experiments. Gremlin increases ECM protein production both in soluble (**b**) and cell-associated fractions (**c**). The same cell number was seed on coverslips in 24-well plates, and grown in 10% FCS medium for 24 h. Then, cells were stimulated with 50 ng/ml Gremlin or 5 ng/ml TGF-β_1 or unstimulated for 48 h in serum-free medium. In the same experiment, the cell-conditioned media was used for evaluation of soluble and the cell-associated ECM proteins. **b** Representative Western blot experiment of soluble fibronectin and type I collagen (lower panel) and at the top the quantification of the data expressed as mean ± SEM of 4 independent experiments. Red Ponceau was used as the loading control of soluble proteins. Additionally, the equal supernatant protein loading was confirmed by the BCA method (not shown). * $p < 0.05$ vs. control. **c** ECM cell-associated proteins were determined by immunocytochemistry. A representative experiment of fibronectin and type I collagen immunostaining of 4 done with similar results.

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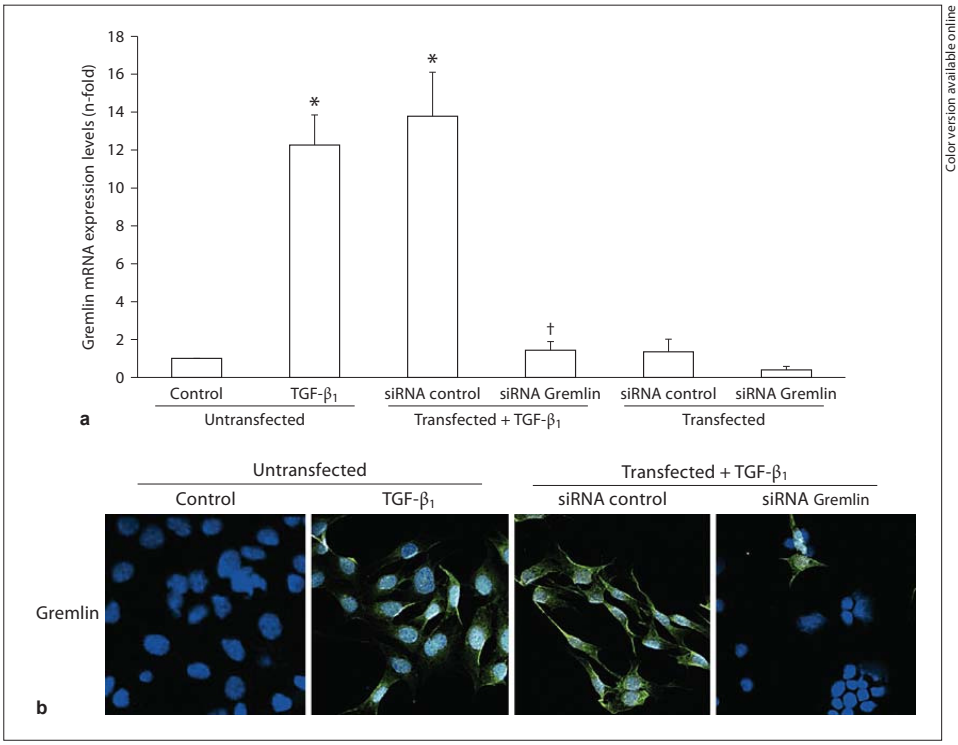

Fig. 2. Gene silencing of Gremlin blocked TGF-β_1-induced Gremlin gene expression and protein production in murine renal fibroblasts. Subconfluent cells were treated with FuGENE (Roche) and left untransfected or transfected for 5 h with 20 nmol/l siRNA of Gremlin or scramble siRNA control. After this time, cells were serum-starved for 16 h before stimulation. Then, cells were treated or not with 5 ng/ml TGF-β_1 for 24 or 48 h for gene or protein studies, respectively. **a** Gremlin gene expression levels, expressed as data mean ± SEM of 7 independent real-time PCR experiments. **b** Gremlin production, determined by indirect immunofluorescence in transfected cells growing on cover slips, as a representative confocal microscopy experiment of 4 done with similar results. These figures show the efficacy of Gremlin silencing at gene (**a**) and protein (**b**) levels. * $p < 0.05$ vs. untreated siRNA control-transfected cells. † $p < 0.05$ vs. TGF-β_1-treated siRNA control cells.

Results

Gremlin Upregulates Profibrotic Factors and ECM-Related Proteins in Cultured Renal Fibroblasts

Stimulation of murine fibroblasts with murine Gremlin for 24 h upregulated the gene expression of several profibrotic growth factors, including TGF-β_1, CCN-2 and PAI-1, and of two key ECM proteins involved in renal fibrosis, fibronectin and type I collagen (fig. 1a). Moreover, Gremlin significantly increased ECM protein levels (both in soluble and cell-associated fractions) after 48 h of incubation (fig. 1b, c). In contrast, stimulation with Gremlin for 24 h did not increase cell proliferation (MTT, not shown; p = n.s. vs. control).

Gremlin Is a Downstream Mediator of Profibrotic Responses to TGF-β in Renal Fibroblasts

Next, we test the hypothesis that Gremlin is a downstream mediator of TGF-β_1-elicited profibrotic responses. Gremlin gene expression was markedly increased after 24 h of TGF-β_1 treatment compared to untreated control cells (both nontransfected cells and siRNA control-transfected cells). In cells transfected with Gremlin siRNA, inhibition of TGF-β_1-induced Gremlin mRNA upregulation (fig. 2a) and protein production (fig. 2b) was observed demonstrating the efficacy of gene silencing, therefore this approach was used to evaluate the role of endogenous gremlin in TGF-β_1-mediated profibrotic responses.

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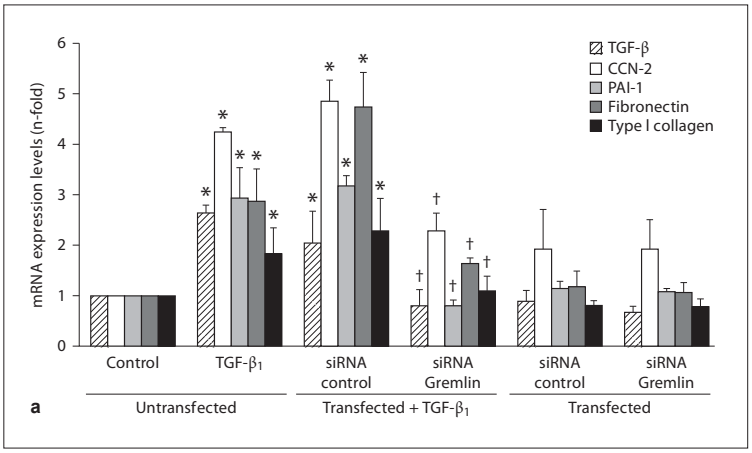


Fig. 3. Silencing of Gremlin prevents the TGF-β_1-induced profibrotic gene overexpression in murine renal fibroblasts. Subconfluent cells were treated with FuGENE (Roche) and left untransfected or transfected for 5 h with 20 nmol/l siRNA of Gremlin or scramble siRNA control. After this time, cells were serum-starved for 16 h before stimulation. Then, cells were treated or not with 5 ng/ml TGF-β_1 for 24 or 48 h for gene or protein studies, respectively. **a** Data of profibrotic gene expression levels, expressed as data mean ± SEM of 7 independent real-time PCR experiments. * $p < 0.05$ vs. untreated siRNA control-transfected cells. † $p < 0.05$ vs. TGF-β_1 treated siRNA control cells.

Gremlin gene silencing downregulated several profibrotic and ECM genes increased by TGF-β_1, compared to TGF-β_1-treated control cells (both nontransfected cells and siRNA control-transfected cells) (fig. 3a). Importantly, silencing of gremlin had no effect on profibrotic and ECM gene expression levels in unstimulated cells, showing that this effect is TGF-β_1 specific. Moreover, Gremlin silencing markedly diminished TGF-β_1-induced upregulation of fibronectin and type I collagen synthesis, both in soluble (fig. 3b) and cell-associated fractions (fig. 3c, d). These data suggest that Gremlin is a downstream mediator of the profibrotic events of TGF-β_1 in renal fibroblasts.

Gremlin Causes EMT in Cultured Human Tubular Epithelial Cells

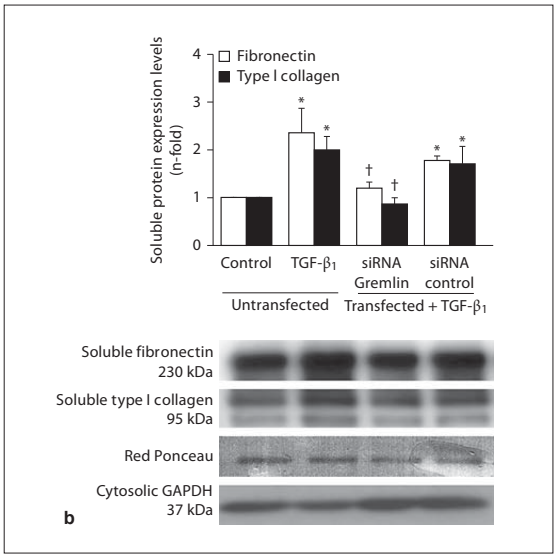
Stimulation of human tubular epithelial cells with Gremlin for 48 h caused phenotypic EMT changes. The transformed cells lost their typical cobblestone pattern of an epithelial monolayer and displayed a spindle-shaped, fibroblast-like morphology (fig. 4a). One of the earliest steps of EMT is the decrease on the expression of proteins that keep basolateral polarity, namely cytokeratin, and intercellular junctions, including the adherent junction protein E-cadherin, that is essential for the structural integrity of renal epithelium [12]. Gremlin downregulated both proteins, cytokeratin and E-cadherin (fig. 4b, c). In unstimulated cells there was no staining for α-SMA and vimentin, two mesenchymal markers, or FSP-1, a specific marker of activated fibroblasts [13], while after Gremlin treatment a positive immunostaining was found (fig. 4b). Gremlin also induced changes at gene levels of EMT-proteins, profibrotic factors (TGF-β_1, CCN-2 and PAI-1), and ECM-related proteins, including MMP-9 (fig. 4d). The upregulation of ECM and MMPs (implicated in basement membranes degradation) is involved in EMT [12]. Gremlin markedly increased fibronectin synthesis (fig. 4e), and MMP-9 activity (fig. 4f). All these data suggest that tubular epithelial cells under Gremlin stimulation undergo a conversion process into myofibroblasts.

Gremlin Is a Downstream Mediator of TGF-β-Induced EMT Changes in Human Tubular Epithelial Cells

In human tubular epithelial cells, the blockade of endogenous Gremlin inhibited TGF-β_1-induced gene upregulation of profibrotic factors, ECM proteins and mesenchymal markers (fig. 5), and markedly inhibited TGF-β_1-induced EMT changes (fig. 5, 6). The efficacy of silencing Gremlin was demonstrated at gene and protein levels (fig. 5a, 6d).

Discussion

Chronic fibrosis represents the final common pathway in progressive renal disease. In normal kidneys there are few resident fibroblasts, but in pathological conditions the number of fibroblasts increases, and they become activated and contribute to ECM synthesis in the tubulointerstitial area, thus inducing fibrosis and the loss of renal function. Our in vitro data demonstrated that in renal



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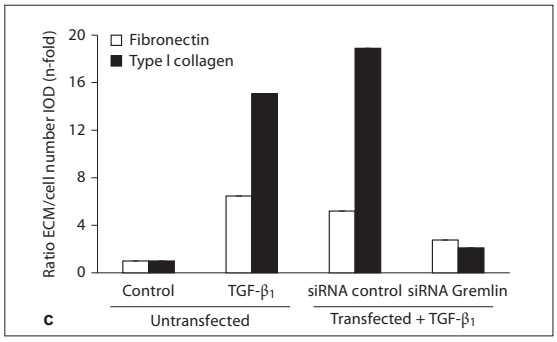
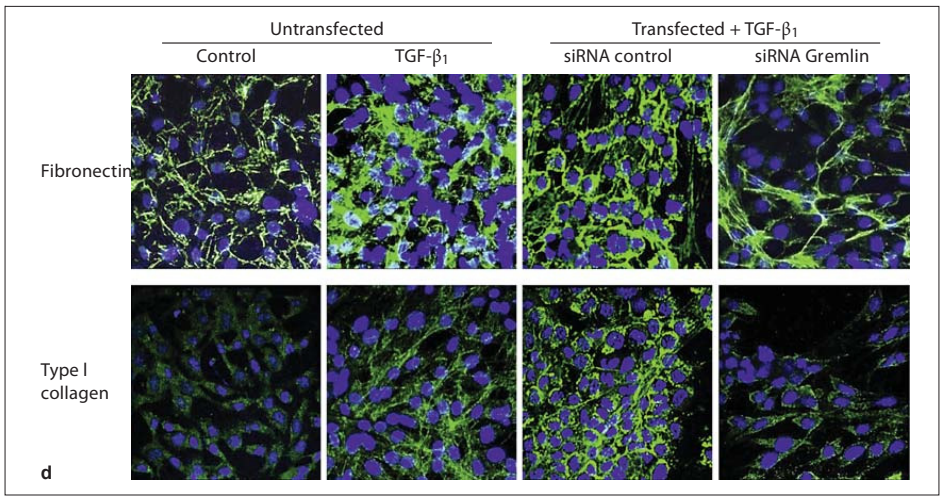


Fig. 3. Silencing of Gremlin prevents the TGF-β_1-induced ECM production of both released (**b**) and cell-associated ECM proteins (**c**, **d**) production in murine renal fibroblasts. Subconfluent cells were treated with FuGENE (Roche) and left untransfected or transfected for 5 h with 20 nmol/l siRNA of Gremlin or scramble siRNA control. After this time, cells were serum-starved for 16 h before stimulation. Then, cells were treated or not with 5 ng/ml TGF-β_1 for 24 or 48 h for gene or proteins studies, respectively. Soluble ECM proteins were quantified in the conditioned media by Western blot. Loading controls used were: Red Ponceau of soluble proteins (to validate sample quality and protein transfer), and GAPDH levels evaluated in total isolated proteins of the corresponding experiments. **b** Representative gel and data as mean ± SEM of 6 independent experiments. * $p < 0.05$ vs. control. † $p < 0.05$ vs. TGF-β_1-treated siRNA control cells. Cell-associated ECM production was determined by indirect immunofluorescence in transfected cells growing on cover slips. Total cell number was evaluated by nuclear staining with 1 μg/mL DAPI (blue staining). **c** Quantification of the ECM immunostaining data, expressed as the ratio of ECM/cell number in integrated optical density arbitrary units. **d** Representative confocal microscopy experiment of 4 done.



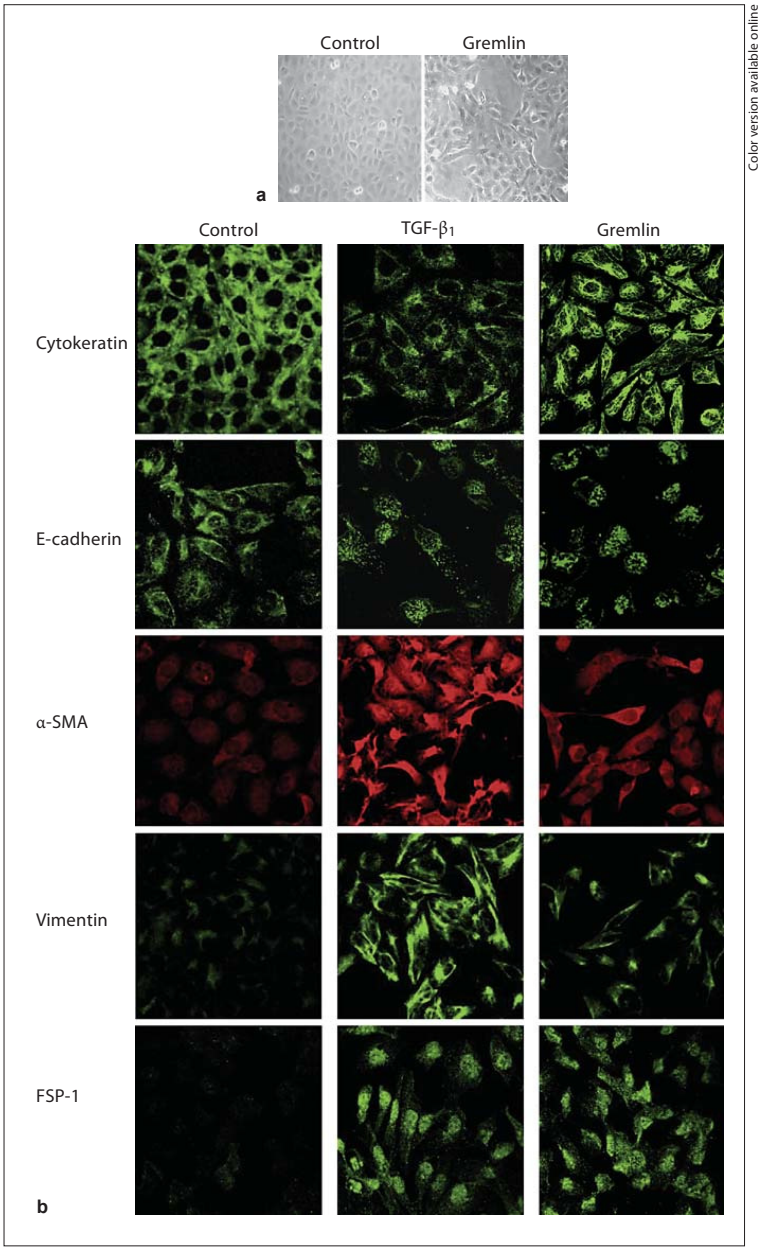


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Fig. 4. Gremlin induces EMT in human tubular epithelial cells. HK2 cells were stimulated with 50 ng/ml Gremlin (human recombinant protein) or 1 ng/ml TGF-β_1 for 48 h and compared to unstimulated cells (control). **a** Contrast phase images show a phenotypic conversion to fibroblast-like shape in response to Gremlin. Magnification ×200. **b** Gremlin induces loss of the epithelial proteins cytokeratin and E-cadherin, and de novo expression of the mesenchymal markers, α-SMA, vimentin and FSP-1. EMT proteins were detected by indirect immunostaining using Alexa 488 and TRITC secondary antibodies. Representative image of 4 confocal microscopy experiments with similar results.

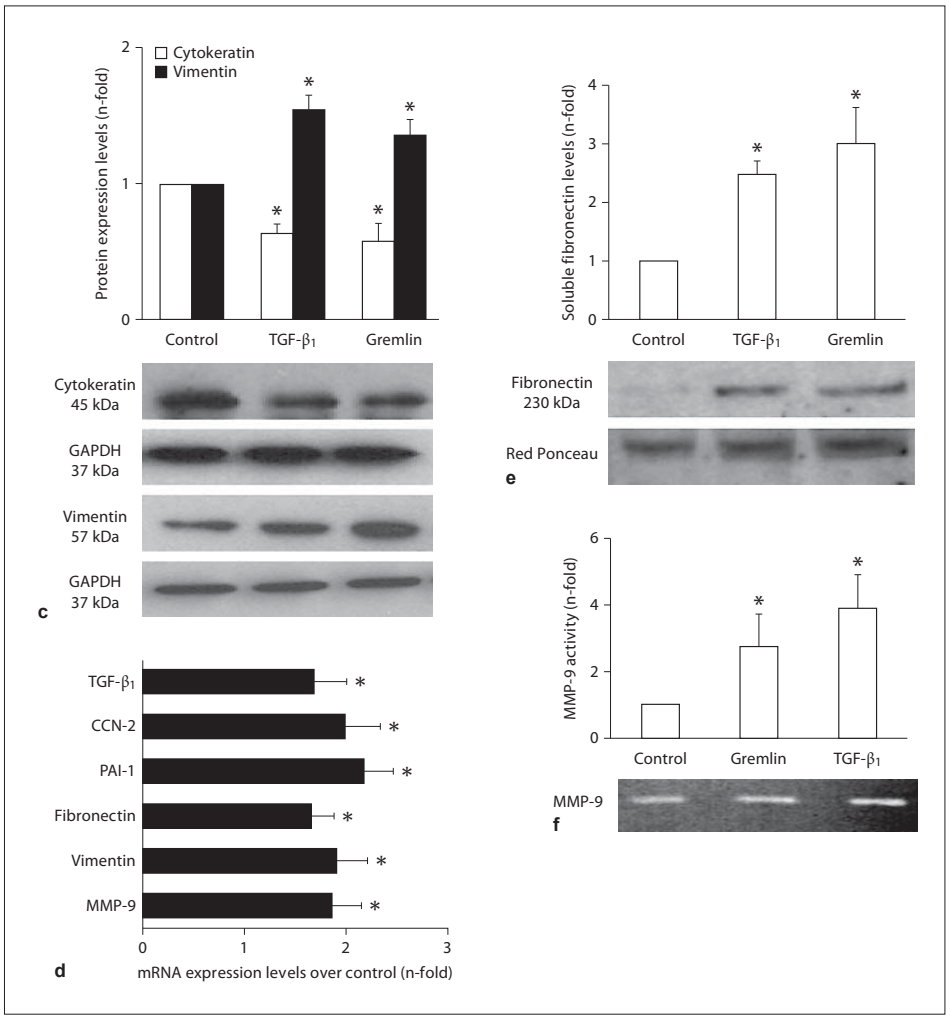

Fig. 4. Gremlin induces EMT in human tubular epithelial cells. HK2 cells were stimulated with 50 ng/ml Gremlin (human recombinant protein) or 1 ng/ml TGF-β_1 for 48 h and compared to unstimulated cells (control). **c** Quantification of EMT changes. Representative Western blot of cytokeratin and vimentin (lower panel), and data as mean ± SEM of 3 independent experiments. Results of total protein expression were obtained from densitometric analysis and expressed as the ratio of protein/GAPDH as n-fold over control. * $p < 0.05$ vs. control. **d** Gremlin modulates gene expression of EMT markers and ECM-related factors. HK2 cells were stimulated with 50 ng/ml Gremlin for 24 h in serum-free medium. Total cell RNA was isolated to assess mRNA levels by real-time PCR. Data are expressed as n-fold over control (considered as 1) as the mean ± SEM of 3 experiments. * $p < 0.05$ vs. control. **e** Gremlin induces the release of fibronectin. Fibronectin was assessed in supernatants from HK2 cells stimulated with Gremlin for 48 h. Red Ponceau was used as the loading control for soluble proteins. Representative Western blot and data as mean ± SEM of 3 experiments are shown. * $p < 0.05$ vs. control. **f** Modulation of gelatinolytic activity of MMP-9 after stimulation with Gremlin for 48 h. Migration properties were determined with the use of standard molecular weight markers. Representative SDS-PAGE zymography that includes a band that corresponds to MMP-9 (□ 90 kDa) and data as mean ± SEM of 3 experiments.

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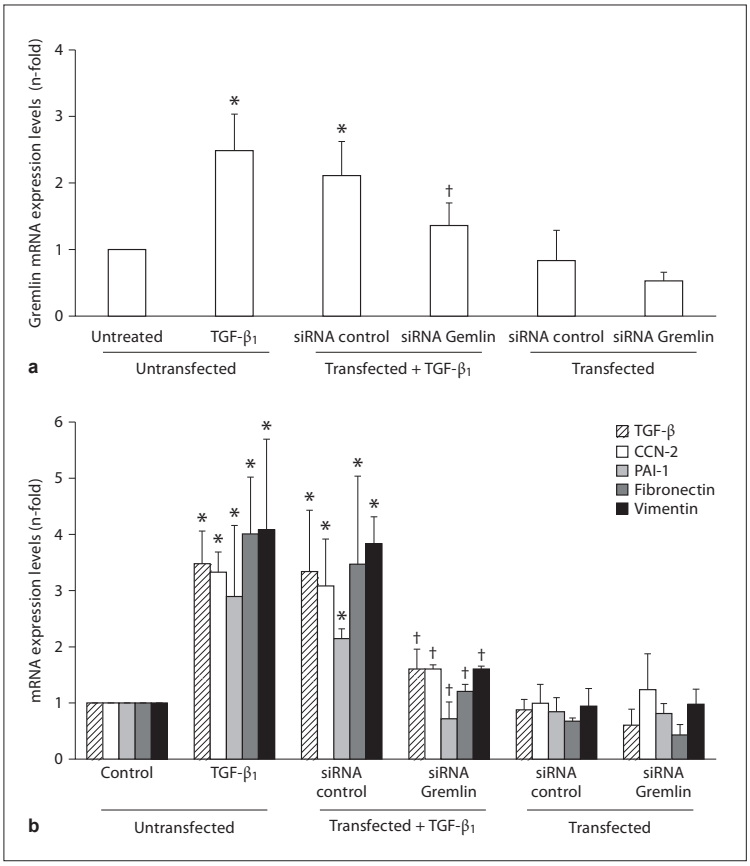

Fig. 5. Effect of Gremlin silencing in TGF-β_1-mediated changes in gene expression of profibrotic-related factors in human tubular epithelial cells. Subconfluent HK2 cells were treated with FuGENE (Roche) and left untransfected or transfected for 5 h with 20 nmol/l siRNA of Gremlin or scramble siRNA control. After this time, cells were serum-starved for 16 h before stimulation. Then, cells were treated or not with 5 ng/ml TGF-β_1 for 24 or 48 h for gene or protein studies, respectively. Figures show gene expression of Gremlin (**a**), profibrotic, ECM factors and vimentin (**b**) determined by real-time PCR. Data are expressed as mean ± SEM of 4 independent experiments. * $p < 0.05$ vs. untreated siRNA control-transfected cells. † $p < 0.05$ vs. TGF-β_1-treated siRNA control cells.

fibroblasts stimulation with Gremlin upregulated the gene expression of profibrotic factors, including TGF-β_1. CCN-2 and PAI-1, and increased the production of type I collagen and fibronectin, both released to the extracellular medium and cell-associated, and therefore Gremlin could contribute to the excessive accumulation of ECM in the tubulointerstitial area, a hallmark of renal fibrosis. Recent data suggest that Gremlin could be an important promoter of fibrosis in different pathologies, including liver fibrosis and lung diseases, pulmonary hypertension and idiopathic pulmonary fibrosis [14–16]. Gremlin also upregulates ECM-related proteins in other cell types, including optic nerve head astrocytes and lamina cribosa cells [17], and, as described here, in cultured interstitial fibroblasts and tubular epithelial cells. All these data suggest that Gremlin could be an important profibrotic factor.

An important source of myofibroblasts could be the injured renal epithelium [1]. Besides the direct activation of fibroblasts, we have demonstrated here that Gremlin could also contribute to fibrosis by inducing EMT in tubular epithelial cells. Gremlin-treated HK2 cells lose their epithelial characteristics, including downregulation of the epithelial proteins E-cadherin and cytokeratin, and changed to a fibroblast-like phenotype characterized by induction of mesenchymal and myofibroblasts markers, α-SMA, vimentin and FSP-1, and activation of MMP-9, a key enzyme involved in the disruption of the epithelial membrane.

TGF-β_1 is the major promoter of EMT during embryogenesis, cancer progression and fibrosis [18]. In fact, adding TGF-β_1 to epithelial cells in culture is a convenient way to induce EMT in various epithelial cells. Now, we have observed that Gremlin induces a similar effect to

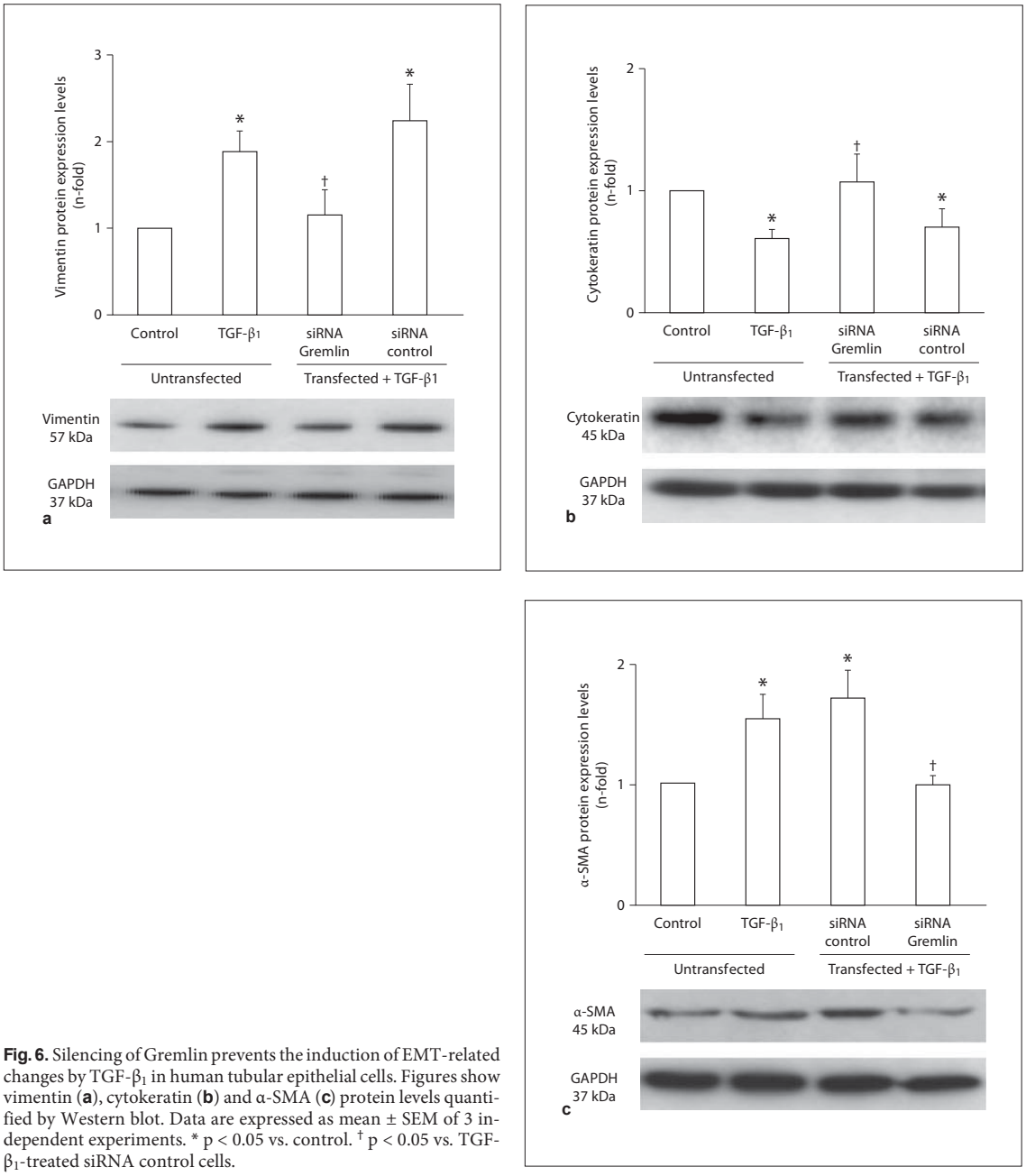

Fig. 6. Silencing of Gremlin prevents the induction of EMT-related changes by TGF-β_1 in human tubular epithelial cells. Figures show vimentin (**a**), cytokeratin (**b**) and α-SMA (**c**) protein levels quantified by Western blot. Data are expressed as mean ± SEM of 3 independent experiments. * $p < 0.05$ vs. control. † $p < 0.05$ vs. TGF-β_1-treated siRNA control cells.

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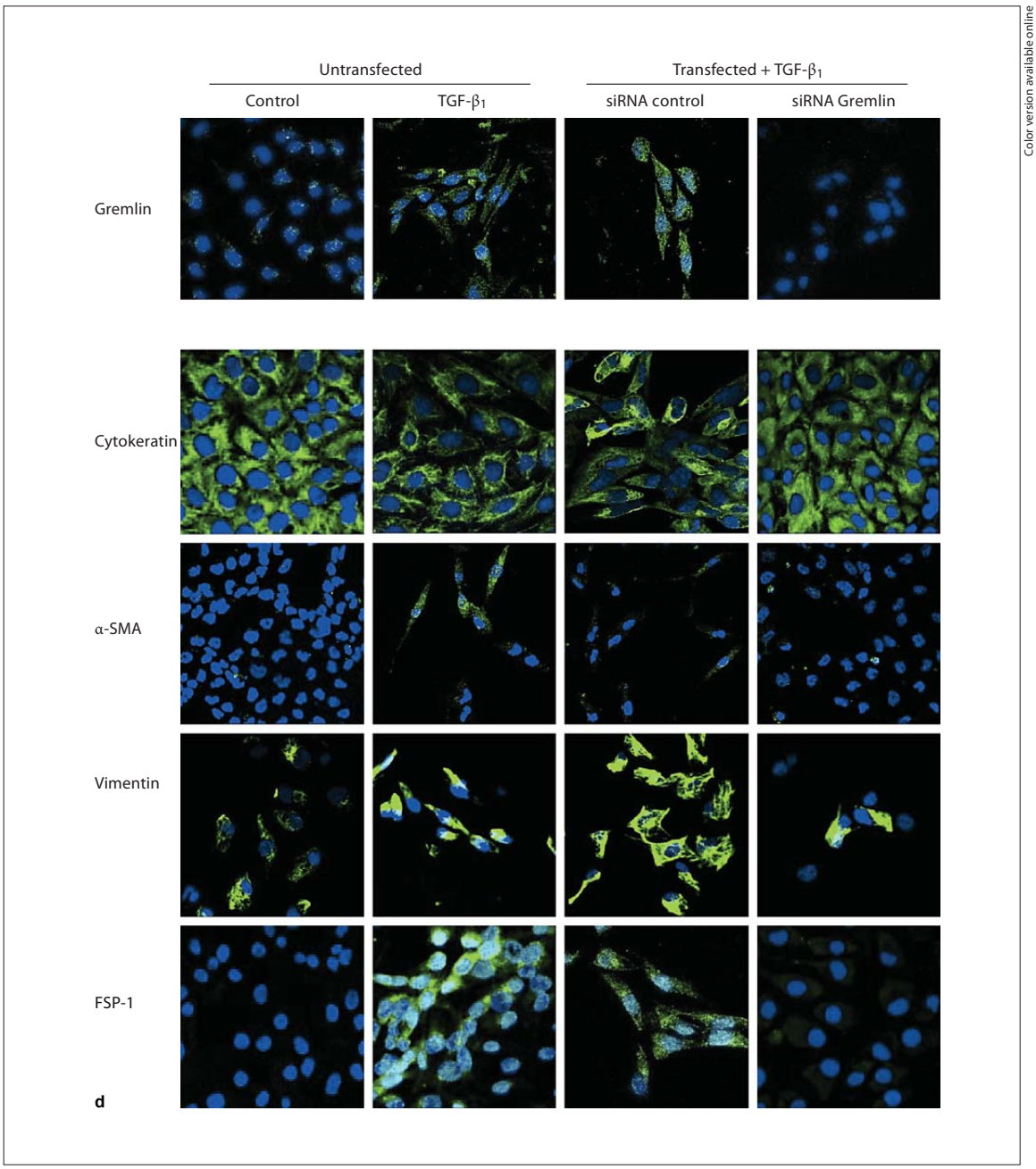

Fig. 6. Silencing of Gremlin prevents the induction of EMT-related changes by TGF-β_1 in human tubular epithelial cells. **d** Representative images of immunocytochemistry for Gremlin and epithelial (cytokeratin) and mesenchymal (α-SMA, FSP-1 and vimentin) markers of 4 confocal microscopy experiments.

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TGF-β_1. In human biopsies of progressive renal diseases, upregulation of Gremlin associated with TGF-β_1 mainly in fibrotic areas has been described [7, 19]. In cultured renal cells TGF-β_1 induces Gremlin expression, including mesangial, tubular epithelial cells [2, 19], and as we show here, fibroblasts. Several authors have suggested a potential interrelation between TGF-β_1 and Gremlin responses. Our in vitro study demonstrates that blockade of endogenous Gremlin by a specific siRNA inhibited overexpression of TGF-β_1-induced profibrotic genes and ECM production in renal fibroblasts. Moreover, Gremlin blockade inhibited TGF-β_1-mediated EMT changes in tubular epithelial cells. Our data suggest that Gremlin could act as a downstream mediator of TGF-β_1-induced fibrosis.

Gremlin heterodimerizes with BMP-2, BMP-4 and BMP-7, preventing their interactions with specific receptors and this capacity is thought to be responsible for the critical role of Gremlin during the process of nephrogenesis, fibrosis and cancer [5]. In asbestos-exposed mouse lungs and pulmonary fibrosis, upregulation of Gremlin was associated with a downregulation of BMP signaling, as demonstrated by the reduced levels of Smad1/5/8 [20, 21]. A similar mechanism has been suggested to be involved in aristolochic acid-induced EMT [22]. BMP-7 and BMP-2 have proven to attenuate TGF-β_1-induced renal interstitial fibrosis by reversing EMT process [23, 24]. However, BMP-independent mechanisms may mediate Gremlin intracellular actions. Several studies have shown direct cellular effects of Gremlin such as its ability to suppress tumorgenesis and modulation of angiogenesis [25, 26], and, as shown here, the regulation of profibrotic factors, matrix proteins and EMT. Studies done in endothelial cells have shown that recombinant Gremlin stimulates cell migration and invasion and triggers tyrosine phosphorylation of intracellular signalling proteins [19, 25]. A recent study in endothelial cells has demonstrated that Gremlin-induced angiogenesis is mediated by binding to VEGFR2, clearly showing a response independent of BMP antagonism [25].

In summary, our study demonstrates for the first time the direct effect of Gremlin in the regulation of fibrotic events in cultured kidney tubulointerstitial cells. Moreover, Gremlin is a downstream mediator of TGF-β_1, a key factor of renal fibrosis. Our therapeutic armamentarium for the treatment of progressive renal disease is limited. Negative results with TGF-β blockers remark the importance of finding novel targets. Our results indicate that Gremlin could be an important modulator of renal fibrosis and open future opportunities for targeting Gremlin as a novel antifibrotic therapy for chronic kidney diseases.

Acknowledgments

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3. Gremlin activa la vía Notch en el riñón

La vía de señalización Notch es un mecanismo altamente conservado, que participa en los procesos de diferenciación, proliferación, y eventos apoptóticos en todas las etapas del desarrollo y tiene un rol crítico durante la nefrogénesis.[5,23,84,134,137] Además, se ha descrito su participación en condiciones patológicas como el cáncer.[184] Una vez que el desarrollo se ha completado, se ha observado una baja activación de la vía Notch en riñón, y se ha descrito su reactivación en un amplio rango de enfermedades renales implicándose en fibrosis túbulo-intersticial.[13,108,116,158,220]

La activación de la vía Notch ocurre a través de la unión del ligando Jagged-1 al receptor Notch-1, que tras el corte proteolítico por acción de la γ-secretasa se libera el fragmento activado de Notch, que migra al núcleo y actúa como un factor de transcripción.[57,88,119] Aproximaciones bioinformáticas han descrito que Gremlin y elementos de la vía de señalización Notch (Jagged-1 ligando de Notch y su efector directo HES-1) comparten elementos regulatorios comunes, en términos de promotor y elementos de unión de microRNA predichos.[245] Sin embargo, los efectos beneficiosos de la modulación de Notch en enfermedad renal todavía es un tema controvertido.[13,67,108]

El objetivo de este trabajo fue determinar si Gremlin podría regular la vía Notch en el riñón y su posible participación en eventos asociados a fibrosis, investigando además el rol de VEGFR2 en estos procesos. En células HK2 en cultivo tratadas con Gremlin, se observó la activación de la vía Notch, caracterizado por incremento en los niveles del ligando Jagged-1 y de la translocación nuclear de Notch-1 activo, pero no observamos aumento de otros componentes de la vía, tales como Delta-1 y Notch-3. La inhibición farmacológica de VEGFR2 con el inhibidor SU5416 o mediante silenciamiento génico, bloqueó la activación de la vía Notch. Los resultados *in vivo*, demuestran que la inyección de Gremlin en el parénquima renal de ratones sanos, activa la señalización Notch en riñón y produce la sobre-regulación de genes pro-fibróticos, incluyendo TGF-β, PAI-1 y componentes de MEC, como Fibronectina y Colágeno tipo I. El tratamiento con SU5416 en ratones inyectados con Gremlin, bloqueó los efectos *in vivo* descritos anteriormente. Asimismo, la inhibición farmacológica de la vía Notch con el inhibidor de la γ-secretasa, DAPT, disminuyó la expresión renal de factores pro-fibróticos. En el modelo experimental de daño renal por UUO se observó activación de la vía Notch a los 5 días,

proceso revertido con el tratamiento con SU5416. Estudios i*n vitro*, en células tubulares muestran que Gremlin induce genes pro-fibróticos (TGF-β y CTGF), proteínas de MEC (Fibronectina) y eventos relacionados a TEM (α-SMA, E-cadherina y Vimentina) vía VEGFR2. Nuestros datos sugieren que Gremlin, a través de VEGFR2, activa la vía Notch lo que se asocia a eventos pro-fibróticos, y por lo tanto podría contribuir a la fibrosis renal.

En estudios previos, en un modelo de UUO, el bloqueo de la vía Notch con el inhibidor de la γ-secretasa, disminuye la expresión génica de Fibronectina y Colágeno tipo I.[13] En este estudio hemos extendido estos datos, demostrando que DAPT disminuye la expresión de genes relacionados a fibrosis, incluyendo TGF-β y PAI-1 y acumulación renal de Fibronectina. Además, en el modelo de administración de Gremlin, DAPT disminuye la expresión de genes pro-fibróticos. En células túbulo-epiteliales en cultivo, DAPT inhibe la expresión de genes asociados a TEM y de factores pro-fibróticos y de MEC. Todos estos datos sugieren que el bloqueo de la vía Notch representa una nueva opción terapéutica para inhibir la fibrosis renal.

Title page

Gremlin activates the Notch pathway in the kidney

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ABSTRACT

The Notch signalling pathway participates in kidney development, but its contribution to renal disease is still a matter of intense debate. Many experimental evidences suggest that Gremlin participates in renal damage and could be a potential therapeutic target for human chronic kidney diseases. A bioinformatics approach has shown that Gremlin and members of the Notch signalling pathway (Jagged-1 and Hes-1) share common regulatory elements. Our aim was to investigate whether Gremlin could regulate the Notch pathway in the kidney and its involvement in fibrogenic associated-events. We have recently described that Gremlin via VEGFR2 induces renal inflammation in mice; therefore the role of VEGFR2 will be also evaluated. In cultured tubular-epithelial cells, stimulation with Gremlin up-regulates gene expression of several Notch components, including Jagged-1 and Notch-1, while Delta-1 and Notch-3 were not changed. Gremlin also increased Jagged-1 production and caused the nuclear translocation of active Notch-1. VEGFR2 blockade, by pharmacological inhibition using the VEGFR2 kinase inhibitor SU5416 or by gene silencing, inhibited Gremlin-induced Notch activation. *In vivo* administration of Gremlin into mice kidney activated the Notch pathway, associated to induction of markers of renal damage and up-regulation of profibrotic genes, including TGF-β. Treatment of Gremlin-injected mice with SU5416 blocked the above *in vivo* Gremlin described-effects. The blockade of Notch activation using the γ-secretase inhibitor, DAPT diminished Gremlin-induced overexpression of profibrotic related genes. In the model of renal damage by unilateral ureteral obstruction blockade of VEGFR2, by SU5416, and of the Notch pathway, by DAPT, diminished renal damage and overexpression of profibrotic factors and ECM-related proteins. In tubular epithelial cells, Gremlin induced profibrotic-related events; including upregulation of profibrotic genes (TGF-β, CTGF), extracellular matrix proteins (Fibronectin) and epithelial to mesenchymal changes, these processes were markedly downregulated by Notch or VEGFR2 inhibition. In conclusion, we propose that Gremlin, via VEGFR2 activates the Notch pathway linked to profibrotic-related events, and therefore could contribute to renal fibrosis.

INTRODUCTION

The Notch pathway is an evolutionarily conserved mechanism, which plays a fundamental role in kidney development and epithelial cell specification.[1] Notch signalling is activated in many biological process including nephrogenesis, tubulogenesis and glomerulogenesis.[2-4] This pathway comprises a family of transmembrane receptors, ligands and transcription factors.[5-7] Notch signalling requires consecutives proteolytic cleavage mediated by ADAM-family metalloproteases and an enzyme complex γ-secretase in Notch to release the intracellular domain (NICD) that acts directly as the biologically active signal transducer.[8]

Many embryological expressed genes regulate morphogenesis and then become quiescent in the normal adult kidney. Notch expression in the kidney is practically absent in healthy adult kidneys,[9] whereas this pathway is activated in various kidney diseases, where the expression of active Notch-1 in tubules is associated with tubule-interstitial fibrosis.[10] However, the contribution of Notch in renal kidney disease is still a subject of discussion.[11]

Among developmental genes reactivated in the adult kidney diseases, Gremlin is an interesting target.[12] Many studies have demonstrated the role of Gremlin in nephrogenesis, mainly acting as a BMP antagonist.[13-15] Inactivation of Grem1 in a homogeneous C57BL/6 genetic background is lethal because of complete renal agenesis.[15,16] In several chronic renal diseases re-expression of Gremlin in the kidney has been described,[10] and some authors have linked Gremlin with diabetic nephropathy.[17-20] We have recently described that Gremlin induces an inflammatory response in the kidney, mediated by the activation of the vascular endothelial growth factor receptor-2 (VEGFR2) signalling pathway.[21] In different pathologies Gremlin is associated to fibrosis. Moreover in several cultured cells, including renal cells, Gremlin regulates profibrotic events and contributes to extracellular matrix accumulation.[22-24] However, the receptor involved and the role of Gremlin in renal fibrosis has not been determined. *In silico* studies have shown that the Notch ligand Jagged-1 and its downstream effector, hairy enhancer of split-1 (Hes-1), shared significant similarity to Gremlin in terms of promoter structure and predicted microRNA binding elements.[25] Therefore, our aim was to evaluate whether Gremlin could regulate Notch signaling pathway in the kidney and its involvement in the fibrotic process, evaluating whether Gremlin acts through VEGFR2.

RESULTS

***Gremlin activates the Notch pathway via* VEGFR2 in cultured tubulo-epithelial cells.**

Cultured tubular epithelial cells (HK2 cell line) were stimulated with human recombinant Gremlin at 10 ng/mL for increasing times. The gene expression analysis of Notch components showed that Gremlin up-regulated the Notch ligand Jagged-1 and its receptor Notch-1 but did not modify Delta-1 and Notch-3 mRNA levels (Figure 1A). Incubation with Gremlin for 48 hours increased Jagged-1 protein levels (Figure 1B). Moreover, by confocal microscopy, Jagged-1 overexpression and nuclear translocation of the active portion of Notch receptor, the Notch intracellular domain (NICD), were detected in Gremlin-treated cells (Figure 1C).

To investigate the contribution of VEGFR2 signaling in Gremlin-induced Notch signaling, HK2 cells were pre-incubated with the VEGFR2 tyrosine kinase inhibitor, SU5416,[26] before stimulation with Gremlin. VEGFR2 inhibition markedly decreased the Gremlin-induced Jagged-1 production, NICD nuclear traslocation (Figure 1B and 1C) and Jagged-1 and Notch-1 gene expression (Figure 1D). By gene silencing the involvement of VEGFR2 in this process was also demonstrated. Transfection of HK2 cells with a small interfering RNA molecule (siRNA) targeting VEGFR2, but not with a nonspecific scramble siRNA, markedly diminished Gremlin-mediated gene overexpression of Jagged-1 and Notch-1 (Figure 1E) and Jagged-1 protein production (Figure 1F).

***Gremlin via VEGFR2* activates Notch pathway in the kidney.**

Next, we investigated whether Gremlin could activate Notch signalling in the kidney. Mice were injected with Gremlin in renal parenchyma and studied at 48 hours. Gremlin up-regulates renal gene expression of Jagged-1 and Hes-1 (Figure 2A) and increased renal protein levels of Jagged-1 (Figure 2B and 2C). In the kidney, Jagged-1 is mainly expressed in tubular cells.[25,27] Immunohistochemistry revealed that Gremlin increased Jagged-1 expression *in vivo* and caused the nuclear translocation of NICD, mainly in tubular epithelial cells (Figure 2D and 2E). The blockade of VEGFR2 with SU5416, blocked Notch pathway activation induced by Gremlin, as shown by down-regulation of gene and protein levels of Notch components to similar levels of control mice (Figure 2A to 2E).

Gremlin increased profibrotic factors gene expression but did not increase matrix proteins accumulation in the kidney.

After 48 hours of Gremlin injection there was no change in renal morphology (as we previously described in paper Gremlin/VEGFR2 presented in this thesis).[21] Now, we have observed that Gremlin-injection increased renal gene expression of some profibrotic factors (TGF-β and PAI-1) and matrix components (Fibronectin and type I Collagen) (Figure 3A). Fibronectin is one of the earliest ECM proteins

upregulated in response to renal injury. However, renal Fibronectin protein production was not increased in Gremlin-injected mice compared to contralateral kidneys (Figure 3B), showing that there was no accumulation of extracellular matrix proteins, at least at the time-point evaluated in this study. The VEGFR2 kinase inhibitor SU5416 diminished Gremlin-induced gene overexpression of profibrotic factors (Figure 3A). Moreover, in Gremlin-injected mice increased cell proliferation was found (Figure 3D) compared to contralateral kidneys.

Treatment with the Notch inhibitor restored Gremlin-mediated renal changes

To demonstrate further the contribution of Notch activation in Gremlin-induced renal responses, mice were pretreated with the γ-secretase inhibitor DAPT that blocks Notch activation components (Figure 2A to 2E). DAPT diminished Gremlin-induced overexpression of profibrotic related genes (Figure 3A and 3B) and biomarkers of renal damage (Figure 3C).

***VEGFR2 blockade inhibits renal* Notch pathway activation and fibrosis in the UUO model**

Gene Array analysis has been demonstrated Notch/Jagged-1 activation in renal cortical epithelial cells exposed to TGF-β.[27] We have recently described that obstructed kidneys presented overexpression of Gremlin associated to VEGFR2 activation.[21] Therefore we have further investigated the relation Gremlin/VEGFR2 and Notch pathway activation in this model.

Five days after UUO, obstructed kidneys showed increased Jagged-1 and Hes-1 gene expression compared with contralateral ones (Figure 4A). Moreover, in obstructed kidneys up-regulation of Jagged-1 protein levels was also found (Figure 4B, 4C and 4D). Activation of Notch was observed by immunohistochemistry, mainly in tubule-epithelial cells (Figure 4C and 4D). In obstructed kidneys from SU5416-treated mice, gene expression and protein levels of Notch pathway components were similar to untreated obstructed ones (Figure 4), suggesting that VEGFR2 is involved in Notch pathway activation.

Obstructed kidneys present tubulo-interstitial fibrosis, as previously described.[28] Therefore, the effect of VEGFR2 signalling inhibition in fibrotic related events was evaluated. In obstructed kidneys of SU5416-treated mice a marked diminution of profibrotic and matrix-related genes were found compared to contralateral kidneys (Figure 5A and 5B). Moreover, renal overproduction of Fibronectin observed in obstructed kidneys was markedly diminished in SU5416-treated mice. These data show that inhibition of VEGFR2 signalling ameliorates renal fibrosis and suggest that blockade of Gremlin/VEGFR2 could be responsible of the downregulation of profibrotic events.

Previous studies have shown that blockade of Notch pathway ameliorates experimental renal damage in several mice models, including in the UUO model.[11] Treatment of obstructed mice with the γ-secretase inhibitor DAPT diminished renal overexpression of profibrotic related genes (Figure 5A) and

Fibronectin production (Figure 5B), to levels similar to contralateral kidneys. These data confirms and extend previous observations.

VEGFR2 activation is involved in Gremlin-induced EMT and fibrotic effects.

Next, we determined whether VEGFR2 is the receptor involved in Gremlin profibrotic responses in tubular epithelial cells. We have previously described that stimulation of tubular epithelial cells with Gremlin induces EMT.[23] One of the earliest steps of EMT is the decrease on the expression of proteins that keep basolateral polarity, and intercellular junctions that are essential for the structural integrity of renal epithelium EMT was also characterized by *de novo* synthesis of mesenchymal markers.[29] HK2 cells were preincubated with VEGFR2 inhibitor (SU5416) before Gremlin stimulation and EMT changes were evaluated by western blot and confocal microscopy. Gremlin induced E-cadherin loss and α-SMA induction was prevented by VEGFR2 inhibition (Figure 6A). VEGFR2 blockade also inhibited Gremlin-induced overexpression of ECM-related (Fibronectin), profibrotic genes (TGF-β1, CTGF) and EMT-related factors (Vimentin) (Figure 6B, 6C and 6D). Finally, we further investigated whether the Notch pathway is involved in Gremlin profibrotic responses. Preincubation with the γ-secretase inhibitor DAPT diminished Gremlin-induced EMT changes as well as gene overexpression of profibrotic and EMC related factors (Figure 6).

All these data indicate that in cultured human tubular epithelial cells Gremlin, via VEGFR2 activation, is capable of regulate profibrotic factors and EMT-related markers, and suggest that Notch pathway is involved, at least in part, in this process.

DISCUSSION

The main finding of this paper is that Gremlin activates the Notch signaling pathway in the kidney, contributing to regulation of profibrotic-related renal events. Several evidences showed some similarities between Gremlin and Notch pathway components. Many studies have demonstrated the involvement of Gremlin and Notch pathway in development, both having an important role during nephrogenesis.[1,15,16,30-32] As occurs with many developmental genes, they are quiescent in normal adult kidney and become activated during pathological changes in adult tissues.[11,17,18,33,34] Our *in vitro* and *in vivo* studies clearly demonstrate that Gremlin increases Notch components in the kidney and activates this signalling pathway by increasing Jagged-1 production. In the kidney, Gremlin causes the nuclear translocation of active Notch and upregulates HES gene expression, the main Notch effector, clearly showing the activation of Notch pathway.

Gremlin acts through its binding to VEGFR2, as demonstrated in renal and endothelial cells (paper Gremlin/VEGFR2 presented in this thesis).[21,35] In cultured tubular-epithelial cells we have found

that VEGFR2 blockade, by pharmacological inhibition using the VEGFR2 kinase inhibitor SU5416 or by gene silencing, inhibited Gremlin-induced Notch activation. Moreover, *in vivo* Gremlin-induced Notch signaling pathway activation was also blocked by VEGFR2 kinase inhibition. These data clearly show that VEGFR2 is the receptor involved in Notch activation caused by Gremlin.

Recent evidences suggest that Gremlin could be an important promoter of fibrosis in different pathologies, including liver fibrosis, lung diseases (particularly pulmonary hypertension and idiopathic pulmonary fibrosis) and myocardial fibrosis.[36-40] In several human renal diseases Gremlin overexpression was found, mainly in areas of tubule-interstitial fibrosis.[18,41-43] Experimental studies in mice have shown that Gremlin blockade diminished renal fibrosis, as observed in streptozotocin-induced diabetes model in knockout mice heterozygous for grem1[19] and by Gremlin gene silencing.[20] Our *in vivo* experiments using a model of parenchymal Gremlin-injection in mice showed a significant increase in fibrotic related genes in the kidney, including TGF-β, that were blocked by the VEGFR2 kinase inhibitor. Moreover, in the model of renal damage by unilateral ureteral obstruction, characterized by renal Gremlin induction, the blockade of VEGFR2 also diminished renal overexpression of profibrotic factors and accumulation of fibronectin, a key ECM protein. These data indicates that Gremlin, via VEGFR2, regulates renal fibrosis *in vitro* and *in vivo*.

Recent studies have demonstrated direct fibrogenic effect of Gremlin in renal cells. In mesangial cells Gremlin increased cell proliferation and ECM accumulation via ERK.[24] In renal fibroblasts Gremlin increased ECM production,[23] including type I Collagen. In tubular epithelial cells Gremlin upregulates profibrotic genes, such as TGF-β and CTGF, and caused EMT changes.[23] Gremlin also induces EMT in airway epithelial cells[44] and in cancer cells.[45] Although the contribution of EMT to renal fibrosis is a matter of intense debate,[46,47] the loss of the epithelial properties of the tubular epithelial cells, including permeability and polarity, may result in decreased viability and contribute to renal injury.[47,48] Therefore, EMT-related changes are an initial step in renal damage and an important potential therapeutic target. Our *in vitro* data demonstrate that Gremlin via Notch pathway regulates EMT in cultured tubular epithelial cells, showing a novel mechanism of Gremlin action in renal cells.

The involvement of Notch pathway in renal damage progression is controversial. In some experimental models of renal damage, including acute kidney injury models, as folic acid administration in mice, Notch blockade ameliorates renal damage, mainly by inhibiting fibroblasts proliferation and therefore decreasing fibrosis. However, a direct effect of Notch in the regulation of fibrotic related events has not been clearly demonstrated. In a previous study in the UUO model, a γ-secretase inhibitor downregulated gene expression of Fibronectin and type I Collagen.[11] We have extend these data showing that DAPT diminished overexpression of profibrotic related genes, including TGF-β and PAI-1 and renal accumulation of Fibronectin. Moreover, in the model of Gremlin administration the γ-secretase

inhibitor DAPT also diminished Gremlin-induced overexpression of profibrotic related genes. In addition, in cultured tubular epithelial cells, DAPT diminished Gremlin-induced EMT changes as well as gene overexpression of profibrotic and EMC related factors. Recently studies have reported that Gremlin induces BMP-independent tumour cell proliferation[49] and cell proliferation in mesangial cells.[24] In this study we have shown that Gremlin administration increased cell proliferation in the kidney. All these data suggest that the blockade of Notch pathway represent a novel therapeutic option to inhibit renal fibrosis.

Future perspectives

Our paper described for the first time that Gremlin activates the Notch signalling in the adult kidney, linked to the regulation of fibrotic-related events, via VEGFR2. Moreover, *in vitro* Gremlin induced EMT is, at least in part, mediated by VEGFR2/Notch activation. Chronic progressive fibrosis of the kidney remains an unresolved challenge. Irrespective of the underlying cause, chronic kidney disease is linked to the development of tubulo-interstitial fibrosis. Our data showing that blockade of Gremlin-mediated VEGFR2/Notch activation ameliorates fibrotic events, suggest that this pathway could be a novel therapeutic target for renal fibrosis, regulating among other process EMT. Although the contribution of EMT to renal fibrosis is a matter of intense debate, this reversal process also participates in embryonic development. Gremlin is a developmental gene that plays a key role in nephrogenesis although the involvement of Gremlin-mediated Notch pathway activation has not been investigated. Future research in renal development to investigate this potential relation is warranty.

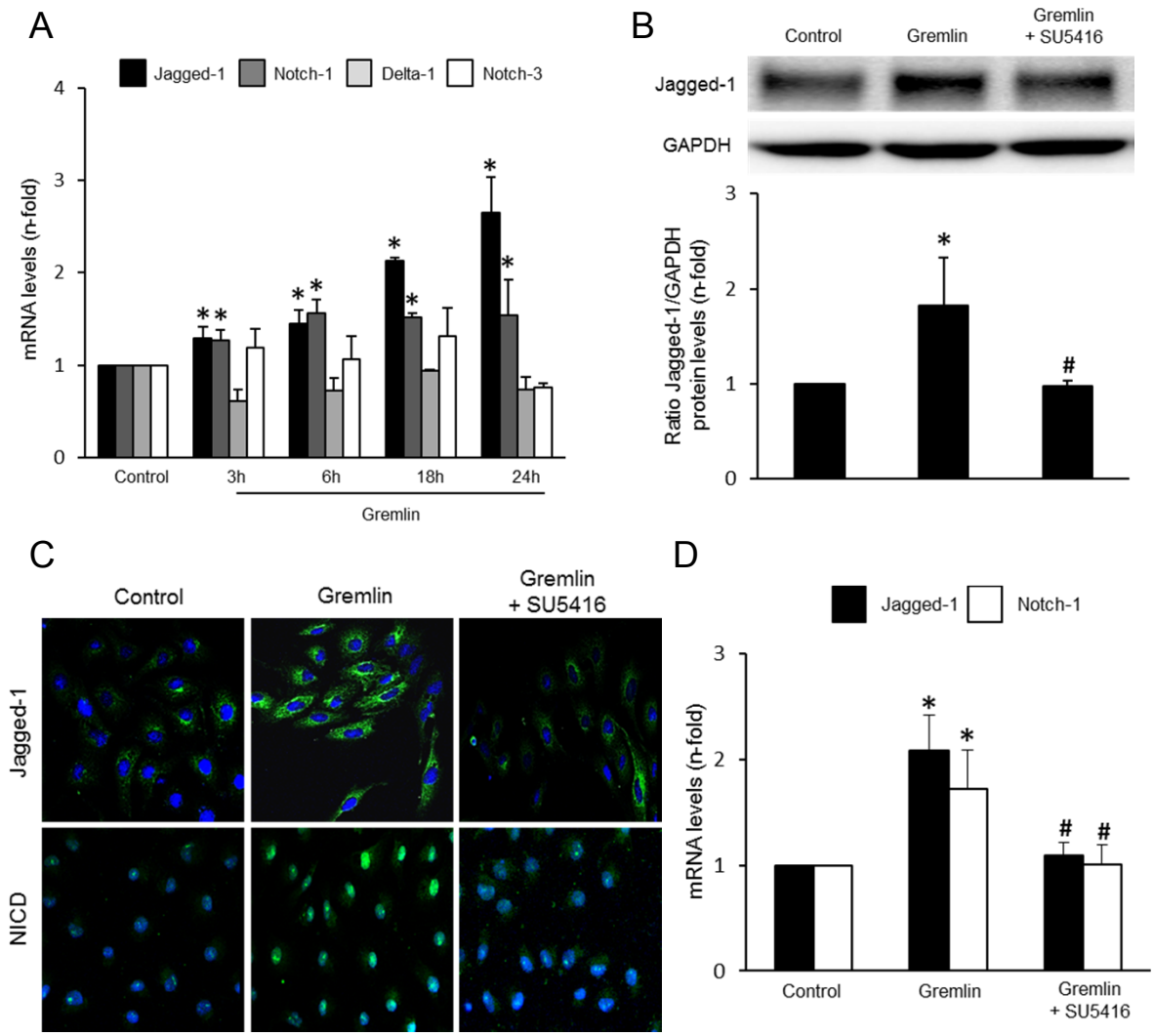

Figure 1. Gremlin activates the Notch pathway via VEGFR2 in cultured human tubular epithelial cells. (A) HK2 cells were treated with 10 ng/ml Gremlin for increasing time periods and Jagged-1, Notch-1, Delta-1 y Notch-3 gene expression was evaluated by real-time PCR. **(B,C,D)** Cells were preincubated with VEGFR2 kinase inhibitor SU5416 at 5 μM for 1 hour and then were treated with 10 ng/ml of human recombinant Gremlin for 48 h. **(B)** Jagged-1 protein levels were evaluated by Western blot analysis in total protein extracts. GAPDH was used as the reference value. **(C) Gremlin causes up-regulation of Jagged-1 and the nuclear translocation of Notch intracellular domain (NICD).** The evaluation of Jagged-1 and NICD expression was done by confocal microscopy with AlexaFluor 488-labeled secondary antibody (green staining). Nuclei were stained with DAPI (blue). Figure shows a representative experiment of 3 done by confocal microscopy. **(D)** Jagged-1 and Notch-1 gene expression was evaluated by real-time PCR. Results are expressed as mean±SEM of 3 independent experiments. *p<0.05 vs control; #p<0.05 vs Gremlin.

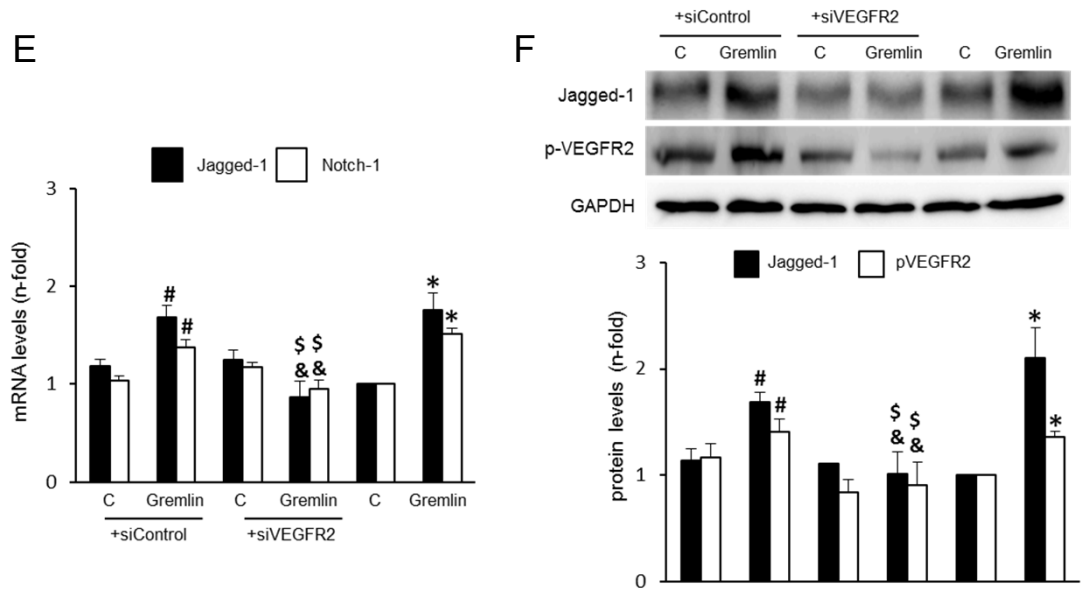

Figure 1. (E,F) VEGFR2 gene silencing blocked Gremlin-induced Notch pathway activation in cultured human tubular epithelial cells. HK2 cells were transfected with a siRNA against VEGFR2 or siRNA scrambled as described in Materials and Methods. Then, cells were stimulated or not with 10 ng/mL Gremlin for **(E)** 24 hours (genes) or **(F)** 48 hours (protein). Data are expressed as mean±SEM of 5 independent experiments. *p<0.05 vs control untransfected; #p<0.05 vs untreated control siRNA-transfected cells; $p<0.05 vs Gremlin-treated control siRNA-transfected cells; &p<0.05 vs Gremlin-treated untransfected cells.

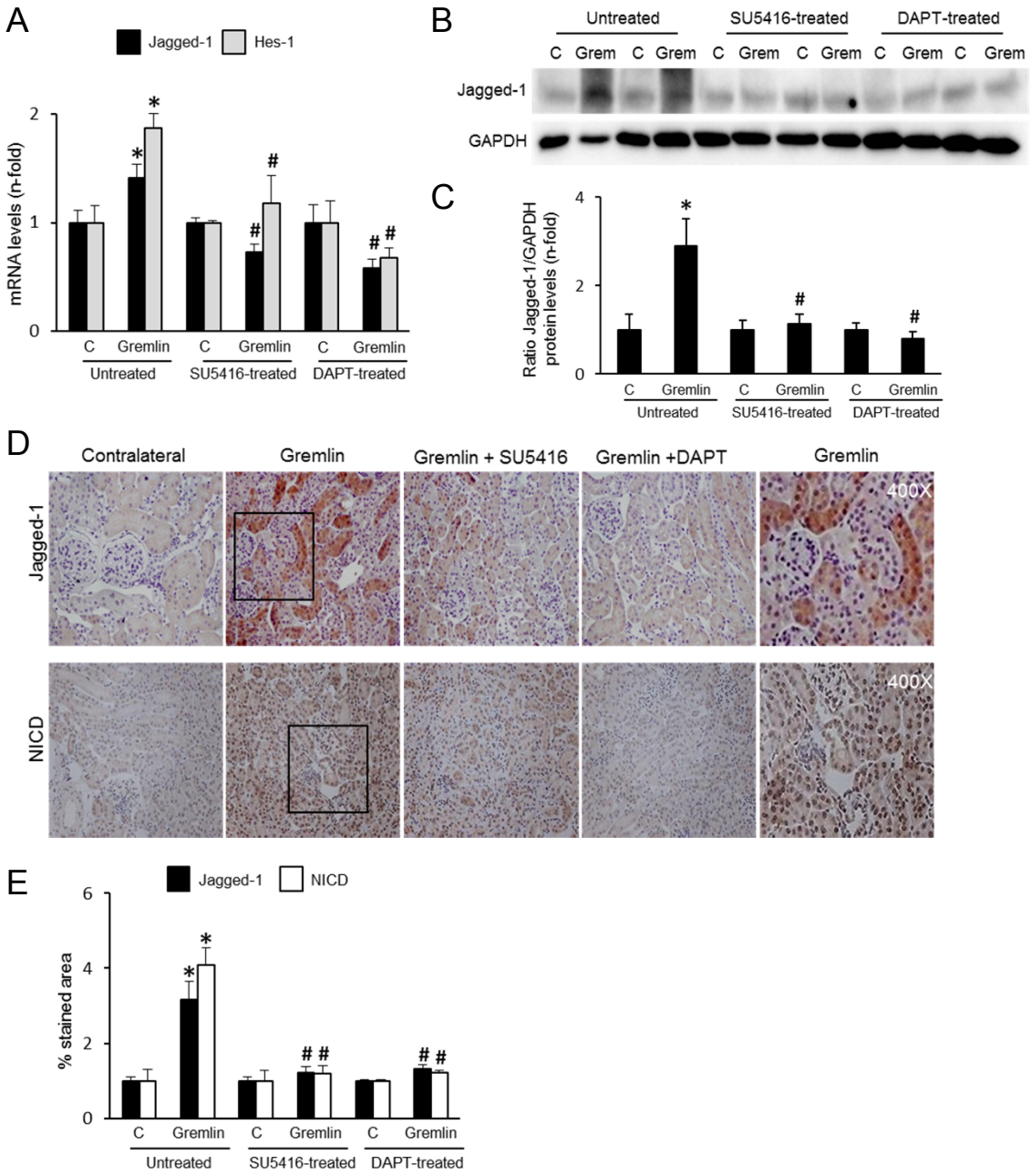

Figure 2. Gremlin activates the Notch pathway via VEGFR2 in the kidney. C57BL/6 mice were injected in renal parenchyma with 50 ng/g of body weight of recombinant Gremlin or vehicle (HCl 4mM) and sacrificed at 48 hours. Some animals were pretreated 24 h before with SU5416 (VEGFR2 kinase inhibitor, i.p; 0.1 mg per mice per day) or its vehicle (0.05% DMSO, control group) before Gremlin administration. **(A)** Gene expression of Jagged-1 and Hes-1 was studied by quantitative real-time PCR. Data are expressed as mean+SEM of 6-8 mice per group. *p<0.05 vs contralateral; #p<0.05 vs Gremlin-injected kidney. **(B)** Jagged-1 synthesis was determined in total renal extracts by Western blot analysis. Figure shows two representative mice from each group. **(C)** Data are expressed as mean+SEM of 6-8 mice per group. *p<0.05 vs contralateral; #p<0.05 vs Gremlin-injected kidney. **(D)** Immunohistochemistry of Jagged-1 and NICD showed a positive immunostaining mainly in tubular epithelial cells. (200x magnification). The right figure show a magnification of 400x **(E)** Quantification of the immunostaining expressed as mean±SEM of 6-8 mice per group. *p<0.05 vs contralateral kidney; #p<0.05 vs Gremlin-injected.

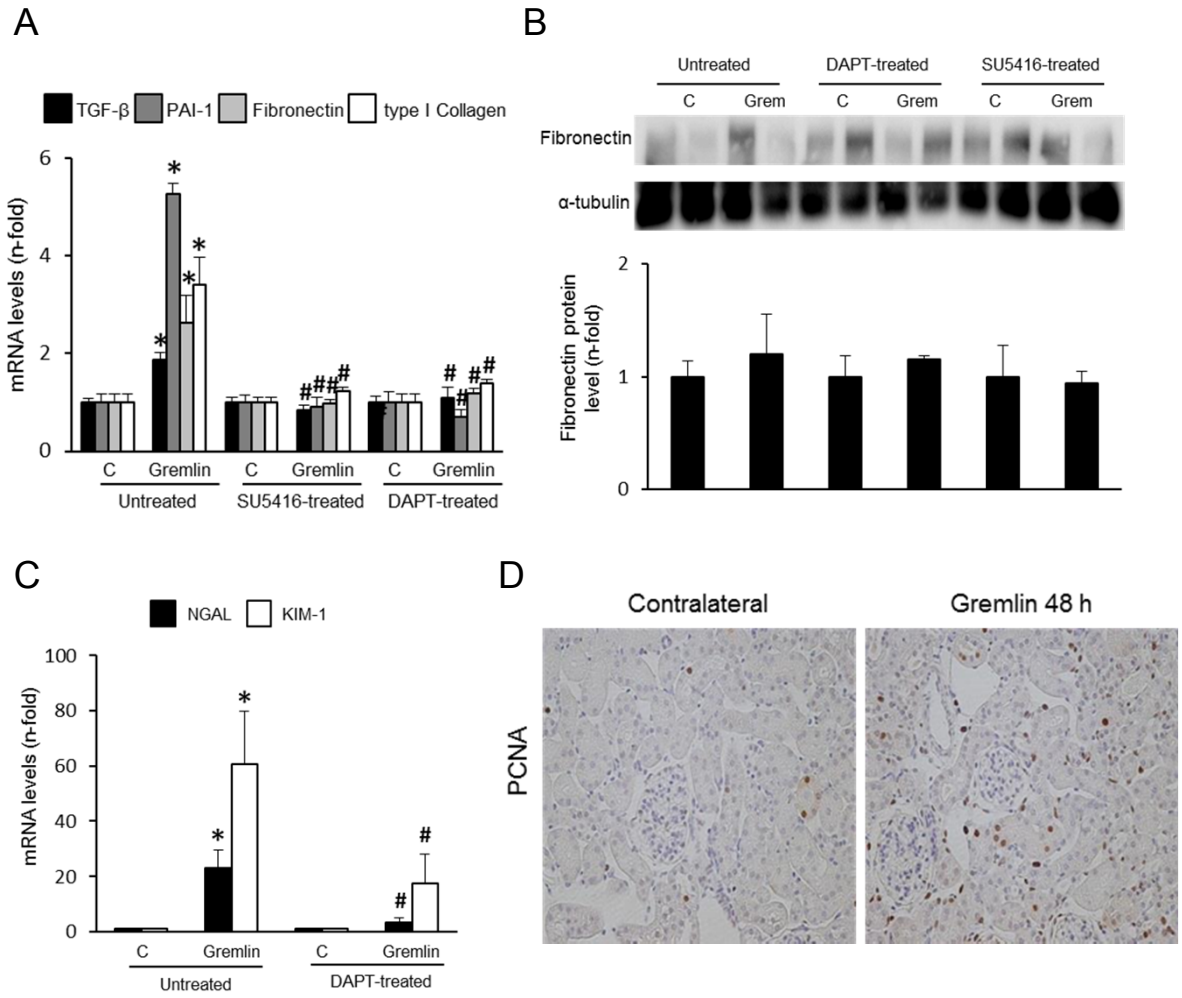

Figure 3. Gremlin increases renal expression of profibrotic factors, matrix components and biomarkers of tissue damage. Effect of VEGFR2 kinase and Notch inhibition. Gremlin-injected mice were daily treated with SU5416 (i.p; 0.1 mg per mice per day) or DAPT (i.p; 0.1 mg per mice per day) starting 24 hours before Gremlin administration. In total renal samples gene expression of TGF-β, PAI-1, Fibronectin and type I Collagen **(A)** was studied by quantitative real-time PCR. **(B)** Renal Fibronectin protein levels were determined by western blot. Equal protein loading was confirmed by probing with anti-α-tubulin **(C)** Damage biomarkers, NGAL and KIM-1 was evaluated by real-time PCR. Results are mean±S.E.M. of 6–8 animals per group, *p<0.05 vs contralateral kidney; #p<0.05 vs Gremlin-injected. **(D)** PCNA expression was evaluated by immunohistochemistry. A representative immunostaining of one mouse of each group is shown (200x magnification).

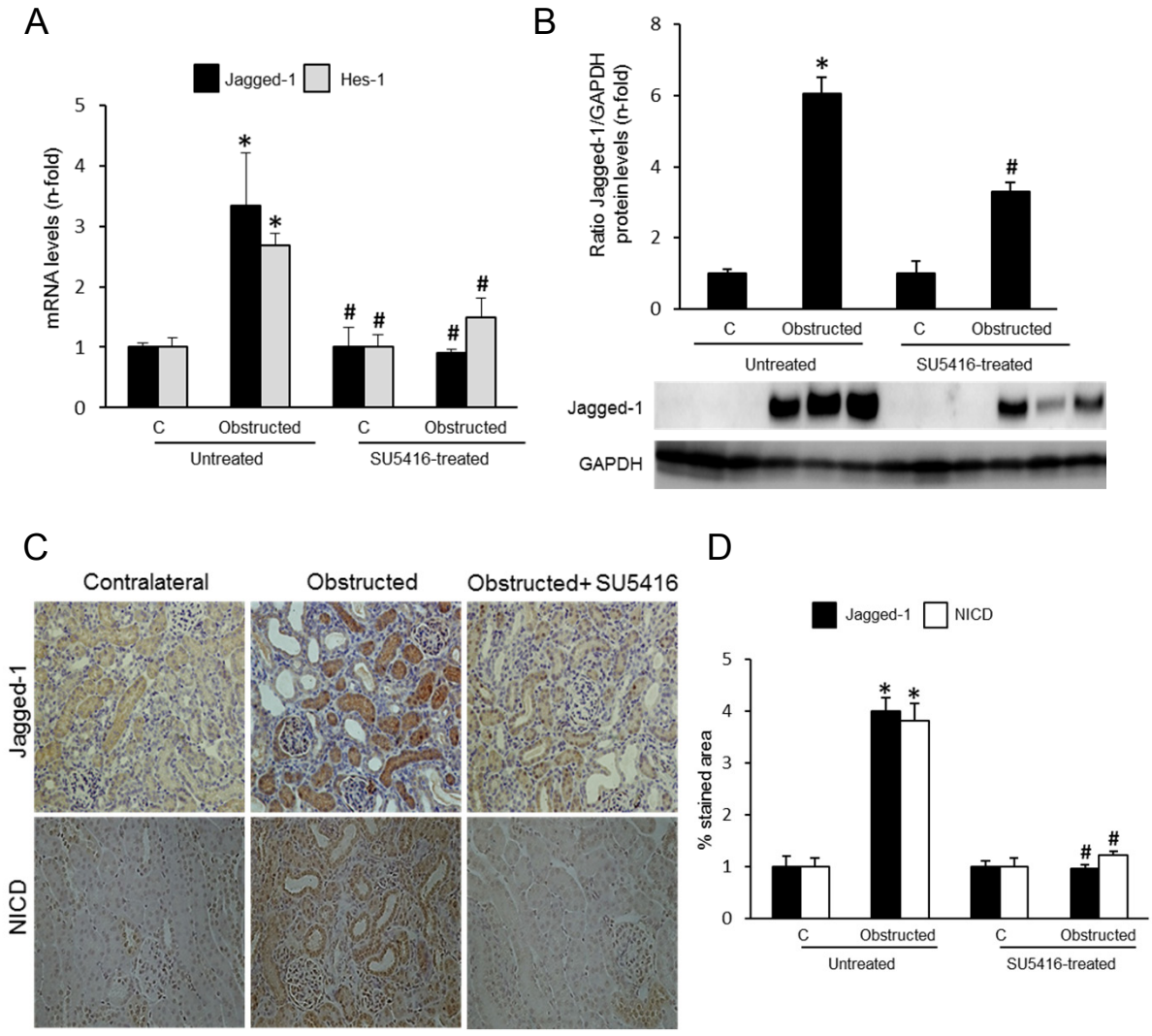

Figure 4. VEGFR2 kinase inhibition blocked the renal Notch pathway activation in the unilateral ureteral obstruction model in mice. (A) Gene expression of Jagged-1 and Hes-1 was studied by quantitative real-time PCR. **(B)** Jagged-1 was assessed by Western blot (a representative blot is shown). Equal protein loading was confirmed by probing with anti-GAPDH. **(C)** Jagged-1 and Notch intracellular domain (NICD) expression were evaluated by immunohistochemistry. A representative immunostaining of one mouse of each group is shown in C (200x magnification), and quantification of data in **D**. Data are expressed as mean±SEM of 6-8 mice per group. *p<0.05 vs control; #p<0.05 vs contralateral kidney.

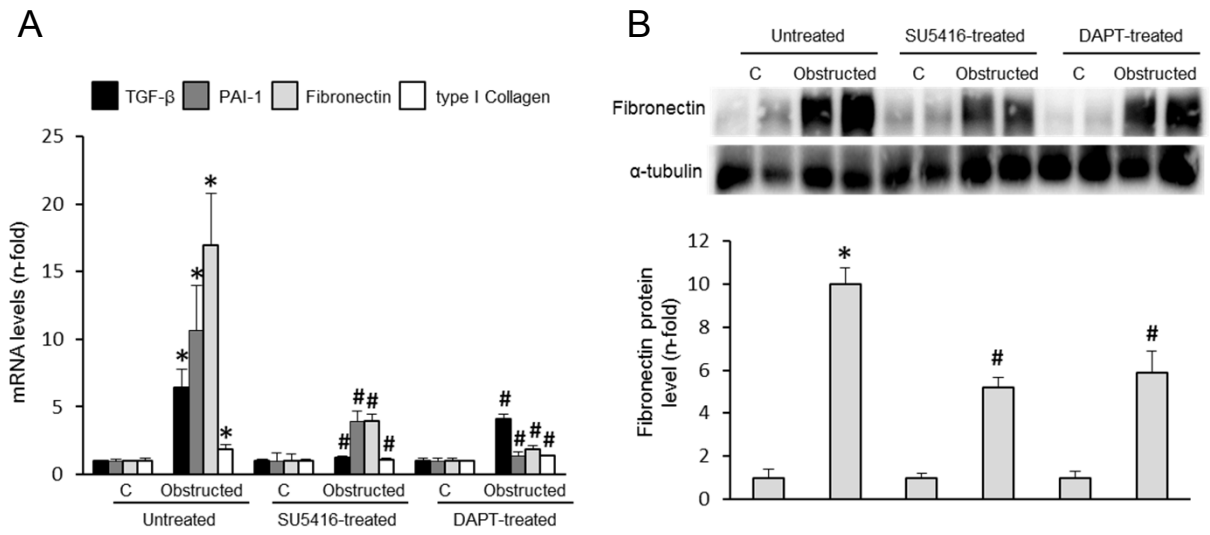

Figure 5. VEGFR2 kinase inhibition or Notch blockade ameliorates experimental renal fibrosis caused by unilateral ureteral obstruction. Mice were daily treated with SU5416 (i.p; 0,1 mg per mice per day) or DAPT (i.p; 0,1 mg per mice per day) starting 24 hours before UUO surgery and studied after 5 days. **(A)** Quantitative real time PCR was performed to determine TGF-β, PAI-1, Fibronectin and type I Collagen gene expression. **(B)** Fibronectin was assessed by Western blot (a representative blot is shown). Equal protein loading was confirmed by probing with anti-α-tubulin. Data are expressed as mean±S.E.M. *p<0.05 vs contralateral; #p<0.05 vs UUO 5 days.

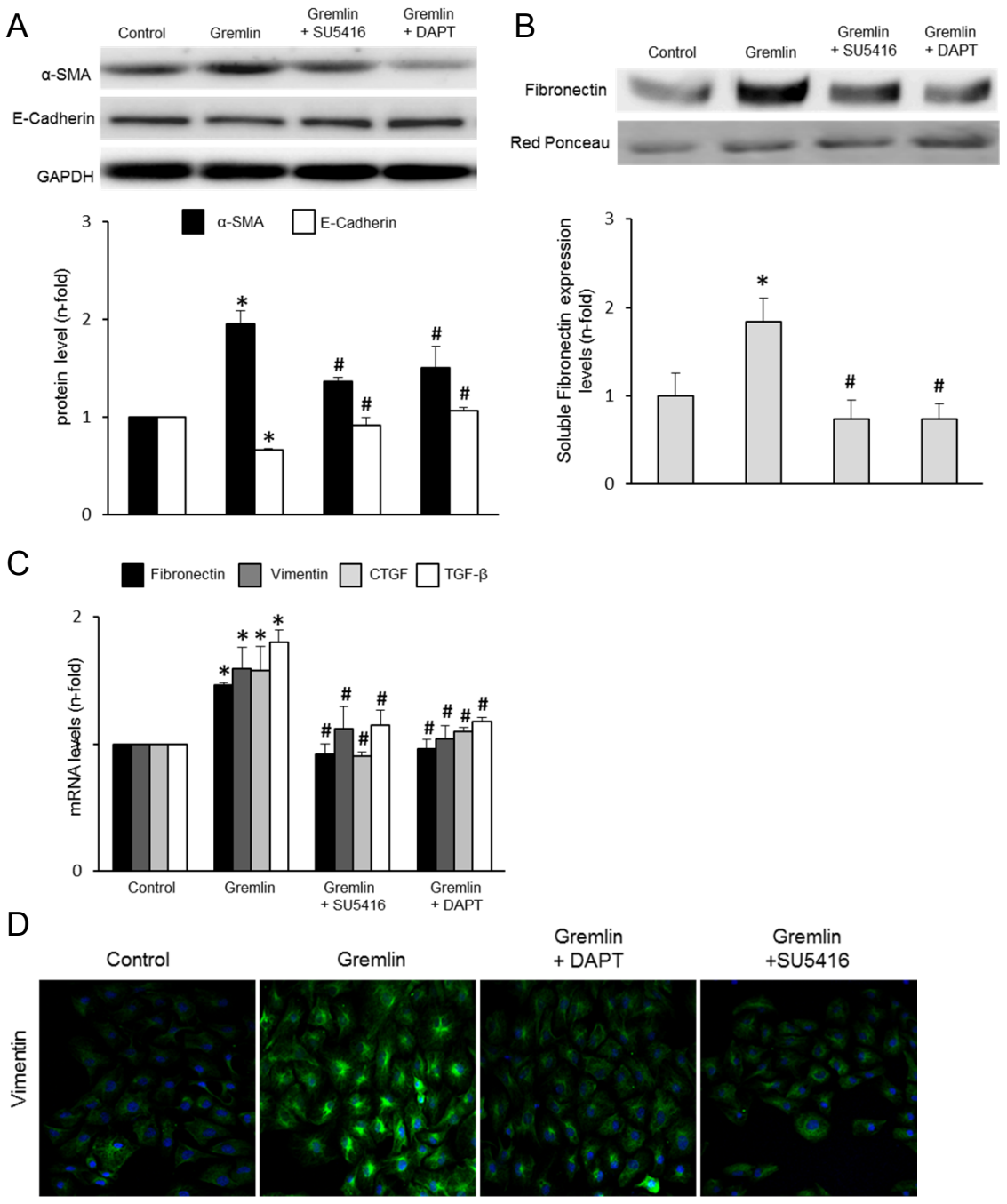

Figure 6. Gremlin induces EMT-changes by activation VEGFR2/Notch pathway in cultured human tubular epithelial cells. Cells were preincubated with SU5416 (5 μM) or DAPT ($3x10^{-8}$ M) before stimulation with Gremlin. **(A)** Gremlin-mediated E-Cadherin loss and α-SMA induction observed after 48 hours was prevented by VEGFR2 or γ-secretase blockade. Figure shows data as mean±SEM of 4 independent experiments. *p<0.05 vs control; #p<0.05 vs Gremlin. **(B)** Figure shows a representative experiment of soluble Fibronectin (lower panel) and in top the quantification of the data expressed as mean±SEM of 5 independent experiments. *p<0.05 vs control, #p<0.05 vs Gremlin. **(C)** Gene expression was evaluated 24 hours after Gremlin stimulation. Total cell RNA was isolated to assess mRNA levels of Fibronectin, Vimentin, CTGF and TGF-β by quantitative real-time PCR. **(D)** Figure shows a representative experiment of 2 done by confocal microscopy.

MATERIALS AND METHODS

Ethics Statement

All animal procedures were performed according to the guidelines of animal research in the European Community and with prior approval by the Ethics Committee of the Health Research of the IIS-Fundación Jiménez Díaz.

Experimental models

The model of intra-renal parenchymal injection of Gremlin was done in 3-month-old female C57BL/6 mice. The model was performed under isofluorane induced anesthesia. Mice received intra-renal-arterial injection of 50 ng of Gremlin recombinant (Cy5 labeled or not) in the left kidney, as described.[50] Mice were studied after 48 hours. The model of unilateral ureteral obstruction (UUO) was done in male C57BL/6 mice. The model was performed under isofluorane-induced anesthesia; the left ureter was ligated with silk (4/0) at two locations and cut between ligatures to prevent urinary tract infection (obstructed kidney), as described.[51] Mice were studied after 5 days. Some animals were daily treated with SU5416 (i.p; 0.1 mg per mice, Vichem, Budapest, Hungary) or DAPT (i.p; 0,1 mg per mice, Calbiochem) starting 1 day before Gremlin-injection or UUO surgery (n= 8 mice per group). At the time of sacrifice, animals were anesthetized with 5 mg/kg xylazine (Rompun, Bayer AG) and 35 mg/kg ketamine (Ketolar, Pfizer) and the kidneys were perfused *in situ* with cold saline before removal. A piece of the kidney (2/3) was fixed, embedded in paraffin, and used for immunohistochemistry, and the rest was snap-frozen in liquid nitrogen for renal cortex RNA and protein studies. In both models, studies were done comparing both kidneys (contralateral and obstructed) in each mouse. In addition, a control group of sham-operated mice was also done, showing the same results than contralateral kidneys (data not shown).

Cell cultured studies

Human renal proximal tubular epithelial cells (HK2 cell line, ATCC CRL-2190) were grown in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml Insulin Transferrin Selenium (ITS) and 36 ng/ml hydrocortisone in 5% CO2 at 37°C. At confluence, cells were growth-arrested in serum-free medium for 24 hours before the experiments. Cells were cultured in six-well plates, serum starved for 24 hours and treated with vehicle or recombinant human Gremlin (Peprotech, 10 ng/mL) for 24 (genes) or 48 (proteins) hours in serum-free medium. In some experiments cells were preincubated for 1 hour with VEGFR2 kinase inhibitor SU5416 (5 µM; Vichem, Budapest, Hungary), or DAPT ($3x10^{-8}$ M, Calbiochem). DMSO, used as solvent, had no effect on cell viability and gene expression (data not shown). Cells were used for protein or RNA studies, and the supernatants (cell-conditioned media) for Fibronectin measurements.

Cell transfection and gene silencing

Gene silencing in cultured cells was performed using either a predesigned siRNA corresponding to the human KDR/VEGFR2 cDNA sequence (s7822; Ambion) or a non-specific control siRNA (Ambion). Subconfluent HK2 cells were transfected for 24 h with Lipofectamine™ RNAiMAX reagent (Invitrogen) according to the manufacturer's guidelines. Then, cells were incubated in serum-free medium for 24 h before the experiments. At some points, cells were treated or not with Gremlin for different times.

Protein studies

For Western blot analysis total protein levels were quantified using a BCA protein assay kit (Pierce), with BSA as the standard. Briefly, equal amounts of proteins (20–100 µg/lane) and conditioned media (30µl) were diluted with reducing sample buffer and separated by SDS/PAGE (8–12% gel) under reducing conditions. Samples were then transferred on to polyvinylidene difluoride membranes (Millipore, Bedford, MA) blocked in PBS containing 0.1% Tween 20 and 5% dry non-fat milk for 1 h at room temperature, and incubated in the same buffer with different primary antibodies overnight at 4ºC. After washing, membranes were incubated with the appropriate HRP (horseradish peroxidase)-conjugated secondary antibody (Amersham Biosciences) and developed using an ECL kit (Amersham Biosciences). The quality of proteins and efficacy of protein transfer were evaluated by Red Ponceau staining (results not shown). Autoradiographs were scanned using the GS-800 Calibrated Densitometer (Quantity One; Bio-Rad Laboratories). The following primary antibodies were employed [dilution]: Jagged-1 ([1:500]; Santa Cruz), phosphorylated-VEGFR2 (Tyr996) ([1:500]; Santa Cruz), α-smooth muscle actin (α-SMA) (1:1000), E-cadherin (1:500), Fibronectin ([1:5000]; BD Pharmigen), α-tubulin ([1/5000]; Sigma) and GAPDH ([1/5000]; Chemicon International).

Paraffin-embedded kidney sections were stained using standard histology procedures. Immunostaining was carried out in 5 µm thick tissue sections. Antigen retrieval was performed using the PTlink system (Dako) with sodium citrate buffer (10 mM) adjusted to pH 6–9, depending on the immunohistochemical marker. Endogenous peroxidase was blocked. Tissue sections were incubated for 1 h at room temperature with 4% BSA in PBS to eliminate non-specific protein binding sites. Primary antibodies were incubated overnight at 4ºC. Specific biotinylated secondary antibodies (Amersham Biosciences) were used, followed by streptavidin–horseradish peroxidase conjugate, and 3,3-diaminobenzidine as a chromogen, then sections were counterstained with Carazzi's haematoxylin. The specificity was checked by omission of primary antibodies. Quantification was made by determining the total number of positive cells in ten randomly chosen fields (×200 magnification) using Image-Pro Plus software. Data are expressed as the positive-stained area compared with the total analysed area. The following primary antibodies were employed: pVEGFR2 (Tyr996) ([1:100], Santa Cruz); Jagged-1 ([1:100]; Santa Cruz), NICD ([1:300]; Abcam), Gremlin ([1:100]; Abgent) and PCNA (Proliferating cell nuclear antigen) ([1:150]; Santa Cruz). For immunocytochemistry experiments, cells were grown on coverslips. After incubation, cells were fixed in paraformaldehyde 4% and permeabilized with 0.2% Triton-

X100 for 10 min. After blocking with 3% BSA for 1 hour, they were incubated with primary antibodies overnight at 4ºC, followed by a AlexaFluor® 488 secondary antibody ([1/300]; Invitrogen) for 1 h. Nuclei were stained with 4′,6-Diamidino-2-phenyindole (DAPI) (Sigma-Aldrich), as control of equal cell density. Absence of primary antibody was used as negative control. Samples were mounted in Mowiol 40-88 (Sigma-Aldrich) and examined by a Leica TCS SP5 confocal microscope. The following primary antibody was employed: Jagged-1 ([1:100]; Santa Cruz), NICD ([1:200]; Abcam) and Vimentin ([1:200]; BD Pharmingen).

Gene expression studies

RNA from cells or renal tissue (pulverized in a metallic chamber) was isolated with TriPure reagent (Roche). cDNA was synthesized by a High Capacity cDNA Archive kit (Applied Biosystems) using 2 µg of total RNA primed with random hexamer primers following the manufacturer's instructions. Next, quantitative gene expression analysis was performed by real-time PCR on an AB7500 fast real-time PCR system (Applied Biosystems) using fluorogenic TaqMan MGB probes and primers designed by Assay-on-Demand™ gene expression products. Human assays IDs were: *JAGGED-1:* Hs01070032_m1; *NOTCH-1*: Hs00413187_m1; *DELTA-1*: Hs01128541_m1, *NOTCH-3*: Hs00194509_m1, *FIBRONECTIN*, Hs00401006_m1 and *VIMENTIN*: Hs00185584_m1. Mouse assays IDs were: *Jagged-1*: Mm00496902; *Hes-1* Mm01342805_m1, *Gremlin*, Mm00483888_s1, *TGF-β*, Mm01178820_m1; *Ngal* (neutrophil gelatinase-associated lipocalin), Mm01324470_m1 and *Kim-1* [kidney injury molecule 1; also known as Havcr1 (hepatitis A virus cellular receptor 1)], Mm00506686_m1. Data were normalized to 18S eukaryotic ribosomal RNA: 4210893E (Vic). The mRNA copy numbers were calculated for each sample by the instrument software using Ct value ("arithmetic fit point analysis for the lightcycler"). Results were expressed in copy numbers, calculated relative to unstimulated cells after normalization against 18S.

Statistical analysis

Results throughout the text are expressed as n-fold increase over control as mean±SEM. Differences between groups were assessed by Mann-Whitney test. Statistical significance was assumed when a null hypothesis could be rejected at $p<0.05$. Statistical analysis was performed using the SPSS statistical software, version 16.0, Chicago, IL).

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4. Angiotensina II contribuye a la fibrosis renal independiente de la activación de la vía Notch

La Ang II participa en la progresión del daño renal contribuyendo a la fibrosis.[200,253] Uno de los tratamientos con mejores resultados demostrados en clínica en patología renal es el empleo de bloqueantes de Ang II, ya que presentan efectos órgano protectores previniendo el desarrollo de fibrosis renal.[4,24] Ang II y TGF-β1 comparten muchos mediadores y sistemas de señalización intracelular implicados en la regulación de fibrosis.[41,201] Estudios en células túbulo-epiteliales en cultivo han demostrado que la activación de Notch está regulada por TGF-β y que media la TEM inducida por este factor pro-fibrótico.[153,169] Sin embargo, se desconoce si Ang II puede regular esta vía de señalización. En este trabajo, nuestro objetivo fue evaluar la contribución del sistema Jagged/Notch en las respuestas renales inducidas por Ang II. En estudios *in vitro*, en células HK2 en cultivo observamos que Ang II no es capaz de aumentar la expresión de genes relacionados con la vía Notch, ni la síntesis de Jagged-1, así como tampoco la translocación de Notch-1 activado al núcleo, presentando una diferencia con la respuesta descrita de TGF-β sobre esta vía. En células HK2 en cultivo, Ang II produce cambios asociados al proceso de TEM (aumento en la expresión del marcador mesenquimal Vimentina y disminución en la expresión del marcador epitelial pan-Citoqueratina). Nuestros resultados muestran que el tratamiento con el inhibidor farmacológico de la γ-secretasa (DAPT) no revierte los cambios causados por Ang II. Respuestas similares obtuvimos en podocitos humanos y en fibroblastos murinos en cultivo. Además observamos que la estimulación con Jagged-1 es capaz de producir cambios asociados a TEM en estas células, lo que demuestra que la vía de señalización Notch participa en la regulación del proceso de TEM. En estudios *in vivo* en modelos experimentales de daño renal, un modelo de ratas espontáneamente hipertensas (SHR) y otro modelo de infusión de Ang II en ratas observamos una mínima expresión de la vía Notch, a pesar de que ambos modelos presentan fibrosis renal. Por otra parte en un modelo de diabetes inducida por la administración de estreptozotocina (STZ) en ratas, apreciamos una activación significativa de la vía Notch asociada a la progresión de daño renal. Estos datos indican que la vía de señalización Notch no está involucrada en daño renal experimental asociado a Ang II y a hipertensión y proporcionan una importante información para entender el complejo rol del sistema Notch en daño renal.

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Angiotensin II Contributes to Renal Fibrosis Independently of Notch Pathway Activation

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Abstract

Recent studies have described that the Notch signaling pathway is activated in a wide range of renal diseases. Angiotensin II (AngII) plays a key role in the progression of kidney diseases. AngII contributes to renal fibrosis by upregulation of profibrotic factors, induction of epithelial mesenchymal transition and accumulation of extracellular matrix proteins. In cultured human tubular epithelial cells the Notch activation by transforming growth factor-b1 (TGF-b1) has been involved in epithelial mesenchymal transition. AngII mimics many profibrotic actions of TGF-b1. For these reasons, our aim was to investigate whether AngII could regulate the Notch/Jagged system in the kidney, and its potential role in AngII-induced responses. In cultured human tubular epithelial cells, TGF-b1, but not AngII, increased the Notch pathway-related gene expression, Jagged-1 synthesis, and caused nuclear translocation of the activated Notch. In podocytes and renal fibroblasts, AngII did not modulate the Notch pathway. In tubular epithelial cells, pharmacological Notch inhibition did not modify AngII-induced changes in epithelial mesenchymal markers, profibrotic factors and extracellular matrix proteins. Systemic infusion of AngII into rats for 2 weeks caused tubulointerstitial fibrosis, but did not upregulate renal expression of activated Notch-1 or Jagged-1, as observed in spontaneously hypertensive rats. Moreover, the Notch/Jagged system was not modulated by AngII type I receptor blockade in the model of unilateral ureteral obstruction in mice. These data clearly indicate that AngII does not regulate the Notch/Jagged signaling system in the kidney, in vivo and in vitro. Our findings showing that the Notch pathway is not involved in AngII-induced fibrosis could provide important information to understand the complex role of Notch system in the regulation of renal regeneration vs damage progression.

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Introduction

The Notch pathway is an evolutionarily conserved mechanism involved in the formation of complex structures, such as the kidney [1–3], that influences differentiation, proliferation, and apoptotic events at all stages of the development [4–6]. The membrane-bound ligands Delta-like-1/3/4 or Jagged-1/2 can bind to the single-pass transmembrane Notch receptors (Notch1/2/3/4) [7,8]. On activation, the Notch intracellular domain (NICD) is cleaved by c-secretases and translocates into the nucleus to interact with the transcriptional regulator recombination signal-binding protein-1 for JKappa (RBP-Jk) and then activate downstream transcription factors, such as hairy-and-enhancer of split 1 (HES-1) and HES-related repressor proteins (Hey), which may mediate the effects on the cell fate [9,10].

The Notch pathway participates in physiological and pathological processes, including cancer [11], and regeneration of the vasculature [12,13]. Regarding the kidney, Notch expression is virtually absent in the glomeruli of healthy adult kidneys, while Notch activation is observed in renal progenitors and podocytes of patients with glomerular disorders [14]. Notch ligands and its receptors are expressed in a wide range of renal diseases. Podocyte-specific Notch expression is correlated with albuminuria and glomeruloesclerosis, while expression of cleaved Notch-1 in tubules is associated with tubulointerstitial fibrosis [15]. In transgenic mice, specific Notch activation in podocytes causes chronic glomerular injury and albuminuria [16,17]. In experimental models of tubulointerstitial damage, activation of Notch is found in tubular epithelial and interstitial cells [5,18,19]. However, the beneficial effect of Notch modulation in renal disease progression is still controversial [14,20,21].

TGF-b1 is a key profibrotic cytokine that contributes to tubulointerstitial damage and renal fibrosis [22–24]. In tubular epithelial cells, TGF-b1 activates the Notch signaling system and stimulates the expression of the Notch ligands Jagged-1, Jagged-2, as well as the receptors Notch-1 and Notch-4, while Notch-2 expression is not affected [18,25]. In these cells TGF-b1 induces

epithelial-mesenchymal transition (EMT) [26]. TGF-b1-induced Jagged-1 overexpression occurs earlier than changes in EMT-associated genes, and blockade of Notch activation markedly diminishes TGF-b1-mediated EMT [27]. Based on these observations, several authors have suggested that the Notch pathway could regulate renal EMT and fibrosis.

Angiotensin II (AngII) plays a key role in the progression of chronic kidney damage, contributing to renal fibrosis. Many in vitro and experimental studies have demonstrated that AngII activates renal cells to produce profibrotic factors and extracellular matrix proteins (ECM) [28,29]. The interrelation between AngII and TGF-b1 is well established. AngII and TGF-b1 share many, profibrotic mediators and intracellular signaling systems [30,31]. In particular, in tubular epithelial cells both AngII and TGF-b1 can induce EMT [23,24,32], and TGF-b1 is known to activate the Notch pathway [18,25]. For these reasons, our aim was to evaluate the contribution of the Notch/ Jagged system to AngII-induced renal responses in this paper. We have identified a signaling mechanism, the Notch pathway, not shared by AngII and TGF-b1, and not involved in AngII-induced fibrosis. Our results may have therapeutic relevance for understanding the complex relation between renal disease progression and regeneration.

Results

AngII did not increase Jagged-1 expression in cultured tubular epithelial cells

In cultured human tubular epithelial HK-2 cells previous studies have shown that TGF-b1, at doses between 5 and 50 ng/ mL, actives Notch pathway and induces EMT changes [27]. Stimulation of HK-2 cells with 102 7 mol/ L AngII did not modify protein levels of the Notch ligand Jagged-1, at any time point studied, while TGF-b1 significantly increased Jagged-1 synthesis, starting at 18 hours and remaining elevated up to 48 hours (ure 1A and B). Moreover, incubation with AngII (dose range 102 6 mol/ L to 102 11 mol/ L) showed no changes in Jagged-1 protein levels (figure 1C). Gene expression analysis of the Notch components showed that only stimulation with TGF-b1, but not AngII, for 24 hours increased Jagged-1 and its receptor Notch-1. In contrast, neither TGF-b1 nor AngII modified Delta-1 and Notch-3 gene levels (figure 1D). By confocal microscopy, activated Notch intracellular domain (NICD) was only detected in the nuclei of TGF-b1-treated cells, while in control or AngII-treated cells there was no NICD immunostaining (figure 1E). These data clearly demonstrated that in tubular epithelial cells TGF-b1, but not AngII, increased the Notch pathway-related gene expression, and activated Notch, determined by Jagged-1 production and NICD nuclear translocation, where it may activate gene transcription, as described [6,33].

Activation of the Notch pathway was not involved in AngII-induced EMT in cultured tubular epithelial cells

In tubular epithelial cells AngII, at the dose of 10^{27} mol/ L, induces EMT changes and upregulation of profibrotic factors and ECM [23,24,32,34,35]. To evaluate the role of the Notch pathway in AngII-induced profibrotic responses cells were treated with the c-secretase inhibitor, DAPT, which inhibits the signaling from all Notch receptor types [6]. Preincubation of HK-2 cells for 1 hour with DAPT inhibited TGF-b1-induced EMT phenotypic changes, including induction of the mesenchymal marker Vimentin and downregulation of the adhesion-related molecule Cytokeratin (figure 2), as described [27]. By contrast, DAPT had no effect on any AngII-induced EMT changes (figure 2). Interestingly, Notch blockade did not modify TGF-b1 or AngII-induced changes in other profibrotic factors, including connective tissue growth factor (CTGF), Matrix metallopeptidase-9 (MMP-9) and Plasminogen activator inhibitor-1 (PAI-1) mRNA upregulation (figure 2D).

We have further explored the direct role of Jagged-1 in EMT. Incubation of HK-2 cells with Jagged-1 recombinant protein for 48 hours induced phenotypic conversion from epithelial to fibroblast-like morphology (data not shown) and changes in EMT markers (figure 3A). DAPT also diminished TGF-b1-induced upregulation of Notch genes (figure 3B) and Jagged-1 production (figure 3C). All these data supporting the hypothesis that TGF-b1 via Notch pathway activation could regulate EMT in tubular epithelial cells.

AngII did not increase Jagged-1 expression in cultured podocytes and renal fibroblasts

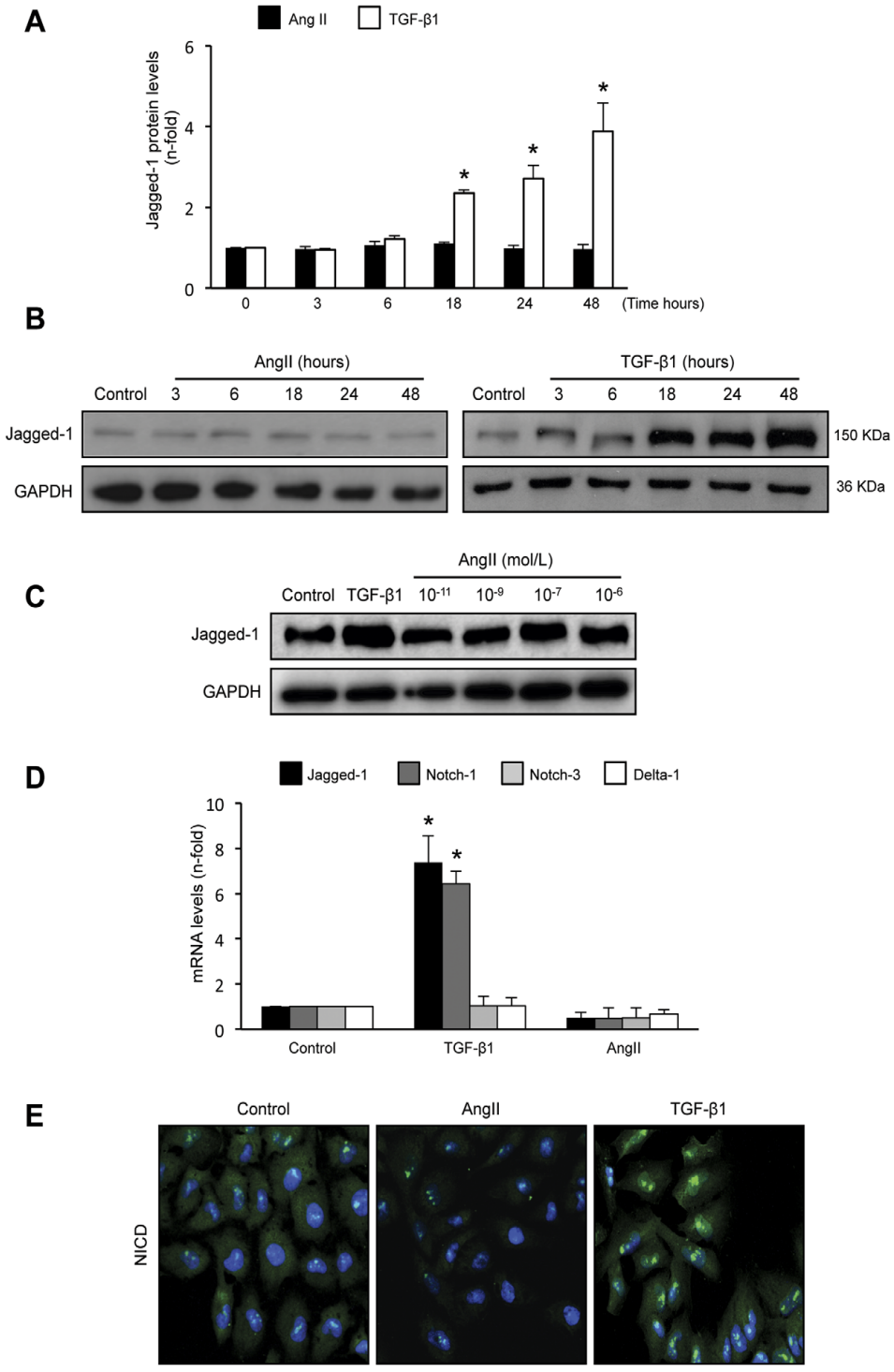
In human podocytes, TGF-b1, but not AngII, upregulated Jagged-1 mRNA (figure 4A) and protein levels (figure 4B). Blockade of Notch activation by DAPT significantly diminished Jagged-1 upregulation by TGF-b1. In murine renal fibroblasts, incubation with TGF-b1, but not AngII, increased Jagged-1 synthesis at 48 hours (figure 4B).

AngII increased TGF-b1 production in renal cells

Previous studies have demonstrated that AngII increased TGF-b1 gene expression and production of active protein [28–32]. Next, the potential role of endogenous TGF-b1 production in the activation of Notch pathway was evaluated. After 48 hours of incubation with 10^{27} mol/ L AngII a significant increase in TGF-b1 production was found in the conditioned media of the different cell types evaluated in the present study (HK-2, TFBs and podocytes) (Figure 5A), in the same experiments that AngII did not active the Notch pathway. The amount of active TGF-b1 detected in supernatants was around 200 pg/ mL. Therefore, we evaluated whether low doses of TGF-b1 could activate the Notch pathway in renal cells. In HK-2 cells, stimulation with TGF-b1 at low doses (2 ng/ mL to 0.5 ng/ mL) did not increase Jagged-1 production, while only doses higher than 5 ng/ mL activated this pathway (figure 5B), as previously described [27]. These data suggest that although AngII increased active TGF-b1 protein levels, this endogenous TGF-b1 production is not enough to activate the Notch pathway in renal cells.

The Notch/Jagged signaling system was not activated in the kidney of AngII-infused rats or hypertensive rats

To investigate the in vivo effect of AngII in the Notch pathway activation in the kidney, the model of systemic infusion of AngII into rats was used. Renal levels of Jagged-1 were not upregulated in response to AngII infusion for 2 weeks compare to saline-infused ones, used as controls (figure 6A and B). By immunohistochemistry, we have confirmed that renal Jagged-1 expression was not changed in response to AngII, at any time point evaluated, up to 2 weeks (figure 6C and D). Notch activation can also be detected by evaluation of NICD levels. In AngII or saline-infused groups NICD immunostaining was similar (figure 6C and D). The onset of renal fibrosis in this model is well characterized. Upregulation of profibrotic genes, including TGF-b1, CTGF and PAI-1, were observed at 3 days. Renal protein levels of CTGF were increased at 3 days, Fibronectin deposition was found 1 week later [36], while TGF-b1 protein levels were not elevated until 2 weeks [32], as we have confirmed here (figure 6E). At 2 weeks sustained overexpression of profibrotic factors and tubulointerstitial fibrosis were observed (data not shown), as described [32,34].


A
Ang II
TGF-β1
Jagged-1 protein levels (n-fold)
0
2
4
6
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6
18
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48
(Time hours)
B
AngII (hours)
TGF-β1 (hours)
Control
3
6
18
24
48
Jagged-1
GAPDH
150 KDa
36 KDa
C
AngII (mol/L)
Control
TGF-β1
10-11
10-9
10-7
10-6
Jagged-1
GAPDH
D
Jagged-1
Notch-1
Notch-3
Delta-1
mRNA levels (n-fold)
0
2
4
6
8
10
Control
TGF-β1
AngII
E
Control
AngII
TGF-β1
NICD

Figure 1. TGF-b1, but not AngII, increased Jagged-1 synthesis in cultured human tubular epithelial cells. Cultured human tubular epithelial cells (HK-2) were treated with 10^{-7} mol/L AngII or 10 ng/mL TGF-b1 for increasing times. A. Results of total protein expression were obtained from densitometric analysis and expressed as ratio protein/GAPDH as n-fold over control as mean 6 SEM of 3 independent experiments. *p, 0.05 vs control. Figure B shows a representative Western blot experiment. C. Dose-response of AngII. HK-2 cells were stimulated with AngII (10^{-6} to 10^{-11} mol/L) for 48 hours and Jagged-1 protein levels were determined by Western blot. Figure shows a representative experiment of 3 done. D. TGF-b1, but not AngII, upregulated Notch-related genes in tubular epithelial cells. Gene expression levels of jagged-1, delta-1 and notch1/3 were determined by Real Time PCR. Data are expressed as mean 6 SEM of 5 experiments. *p, 0.05 vs control. E. Nuclear localization of activated Notch-1 (NICD) is only observed in TGF-b1 treated cells for 48 hours (green staining), while in control and AngII-treated cells there is no positive NICD staining. Nuclei are in blue (DAPI staining). Figure shows a representative experiment of 2 done by confocal microscopy. Magnification 200x.
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We further evaluated the role of Notch pathway in hypertension-induced renal damage using the model of spontaneously hypertensive rats (SHR). At 16 weeks of age, SHR rats presented elevated blood pressure, increased proteinuria and urinary albumin (Table 1), TGF-b1 overproduction (figure 6E) and elevated collagen deposition (data not shown), compare to control WKY of the same age. In SHR, renal Notch pathway was not activated, shown similar levels that normotensive WKY rats

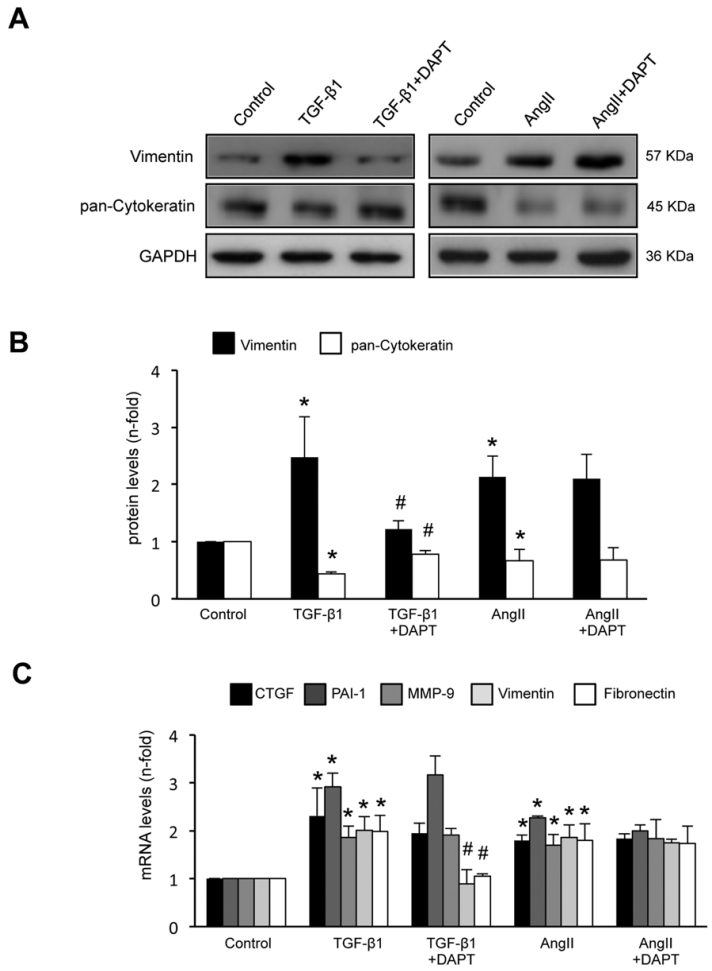

Figure 2. Blockade of the Notch pathway inhibited TGF-b1-, but not AngII- induced EMT changes in cultured tubular epithelial cells. HK-2 cells were pretreated with the gamma-secretase inhibitor, DAPT (36 10^{-8} mol/L) for 1 hour and then stimulated with 10^{-7} mol/L AngII or 10 ng/mL of TGF-b1 for 24 or 48 hours (gene and protein studies, respectively). Figure A shows a representative Western blot experiment B. Results of total protein expression were expressed as mean 6 SEM of 3 independent experiments. Figure C shows gene expression levels, determined by Real Time PCR, were shown as mean 6 SEM of 5 experiments. *p, 0.05 vs control, # p, 0.05 vs TGF-b1.
doi:10.1371/journal.pone.0040490.g002

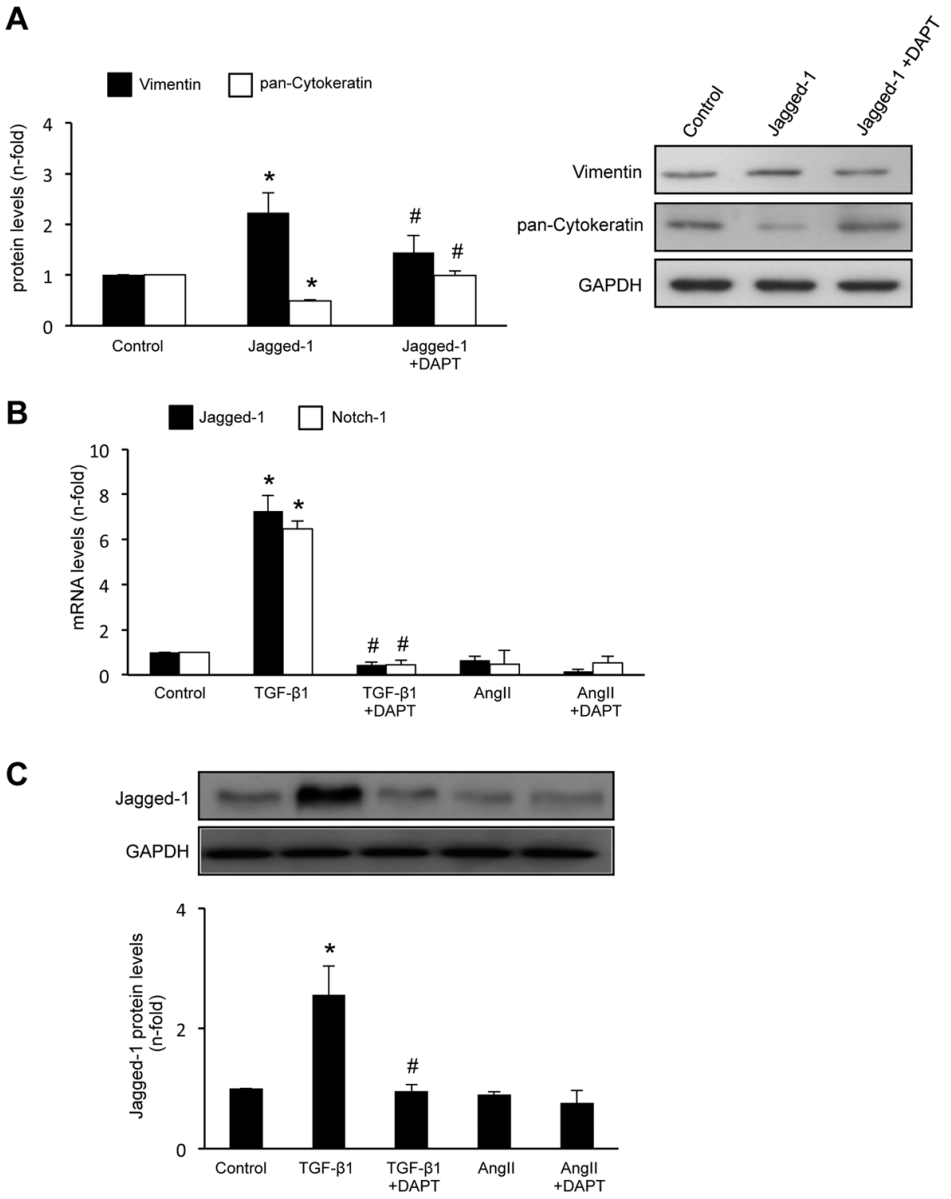

Figure 3. Jagged-1 induced EMT changes in cultured tubular epithelial cells. A. HK-2 cells were treated with 50 ng/mL Jagged-1 for 48 hours. Left panel: total protein levels as mean 6 SEM of 3 independent experiments. *p, 0.05 vs control; # p, 0.05 vs Jagged-1. Right panel: representative Western blot experiment. Blockade of the Notch pathway inhibited TGF-b1-induced upregulation of Notch components. Cells were pretreated for 1 hour with 36 10^{-8} mol/L DAPT and then incubated with 10 ng/mL TGF-b1 or 10^{-7} mol/L AngII for 24 or 48 hours (gene and protein studies, respectively). B. Gene expression levels are expressed as mean 6 SEM of 5 experiments. Figure C shows a representative western blot of Jagged-1 and data as of mean 6 SEM of 3 independent experiments. *p, 0.05 vs control; # p, 0.05 vs TGF- b1.
doi:10.1371/journal.pone.0040490.g003

(figure 6), as described in hypertensive patients with renal injury [15].

An additional control of the experiment was to evaluate whether renal Notch is activated in a rat model of diabetic nephropathy induced by streptozotocin injection (STZ). Previous studies have demonstrated activation of renal Notch in human and experimental diabetic nephropathy [16,37]. At 6 weeks after induction of diabetic nephropathy, rats presented increased proteinuira and

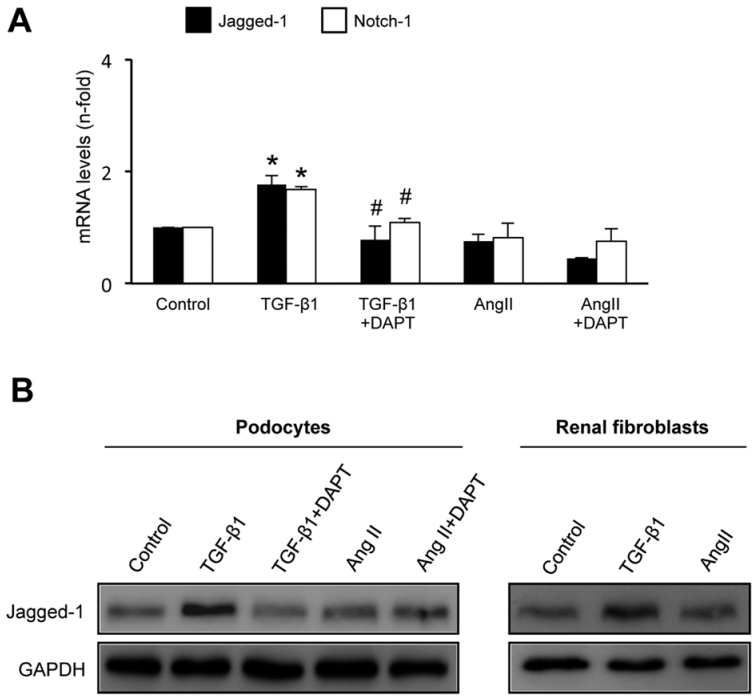

Figure 4. TGF-b1, but not AngII, increased Jagged-1 expression in human podocytes and murine renal fibroblasts. Cells were treated with 10^{-7} mol/L AngII or 10 ng/mL TGF-b1 for 24 or 48 hours (gene and protein studies, respectively). In some points cells were pretreated with the gamma-secretase inhibitor, 36 10^{-8} mol/L DAPT, for 1 hour. A. In human podocytes, gene expression levels of Notch components are expressed as mean 6 SEM of 5 experiments. *p, 0.05 vs control, # p, 0.05 vs TGF- b1. B. Representative Western blot of Jagged-1 levels in podocytes and fibroblasts of 3 independent experiments.
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albuminuria (Table 1), elevated renal TGF-b1 protein levels (figure 6E) and fibrosis (not shown). In the immunohistochemistry experiments done in parallel with the other models, the renal samples of the diabetic rats showed a clear up-regulation of Jagged-1 and NICD levels, mainly in tubulointerstitial and glomerular cells (figure 6).

Blockade of AngII receptors did not regulate Notch/ Jagged signaling system in the model of unilateral ureteral obstruction in mice

Several groups have shown activation of Notch/ Jagged in experimental models of renal damage. Interestingly, microarray analysis discloses that Jagged-1 is one of the most highly expressed genes in the experimental model of unilateral ureteral obstruction (UUO) [5,18,19]. Previous studies have demonstrated that AngII plays a key role in the pathogenesis of UUO, and pharmacological blockade of AngII (by ACE inhibitors or AT1 receptor antagonists) ameliorates disease progression [38,39]. However, there are no studies evaluating whether AngII blockade modulates the Notch pathway in experimental renal diseases. Thus, in UUO model, we have observed that treatment with an AT1 antagonist (losartan, 10 mg/ kg/ day), ameliorated disease progression, including inhibition of inflammatory cell infiltration and downregulation of MCP-1 gene overexpression (figure S1), and diminution of renal fibrosis (not shown) and TGF-b1 overproduction (figure S1), as previously described [38,39]. Jagged-1 protein levels were markedly increased in obstructed kidneys compare to contralateral ones, as described [5,18,19]. In losartan-treated mice, obstructed Jagged-1 protein levels were similar than in untreated obstructed ones (figure 7). This data further supports the notion that AngII regulates renal fibrosis independently of Notch pathway.

Discussion

The main finding of this paper is that AngII does not modulate the Notch pathway in the kidney. In in vivo studies, we have found that systemic infusion of AngII into rats for 2 weeks, at a dose that caused tubulointerstitial damage and fibrosis, did not upregulate renal expression of activated Notch or Jagged-1, suggesting that the Notch/ Jagged pathway is not involved in AngII-induced renal damage. In spontaneously hypertensive rats, studied at the time that presented albuminuria and interstitial fibrosis, renal expression of activated Notch or Jagged-1 was similar to normotensive healthy WKY rats of the same age. In a wide range of kidney diseases, renal activation of the Notch components has been described. However, these authors have observed that in patients with hypertensive nephrosclerosis renal the Notch/ Jagged-1 system was not upregulated [15]. Blockade of AngII, by ACE inhibitors or receptor blockers, is one the current clinical therapies that have proven to ameliorate renal disease progression [31]. In the experimental model of UUO, we have found that AT1 antagonist treatment ameliorated renal inflammation and fibrosis,

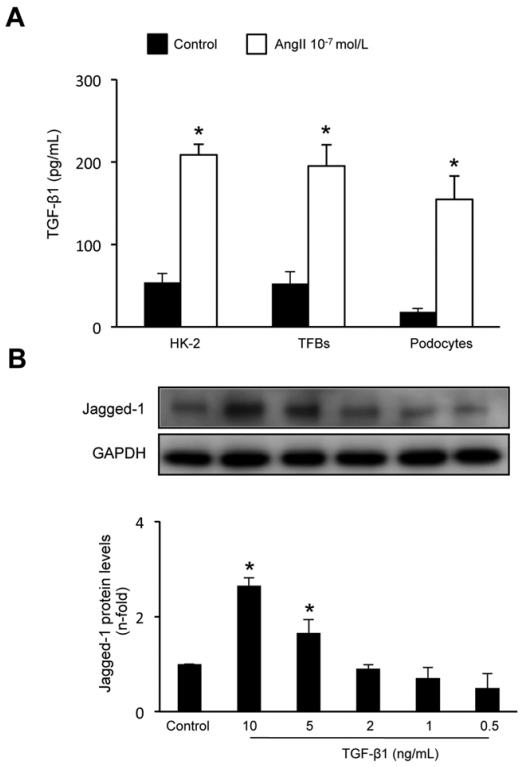

Figure 5. AngII increased TGF-b1 production in renal cells. The different cell types, human tubular epithelial cells (HK-2), murine renal fibroblasts (TFBs) and human podocytes, were treated with 10^{-7} mol/L AngII for 48 hours. Then, supernatants were collected, and active TGF-b1 was determined by ELISA. Figure A shows TGF-b1 protein levels as mean 6 SEM of 3 independent experiments analyzed by duplicate. *p, 0.05 vs control. B. Low doses of TGF-b1 did not increase Jagged-1 protein production in tubular epithelial cells. HK-2 cells were stimulated with TGF-b1 (10 to 0.5 ng/mL) for 48 hours and Jagged-1 protein levels were determined by Western blot. Figure shows a representative experiment and data as mean 6 SEM of 3 experiments. *p, 0.05 vs control.
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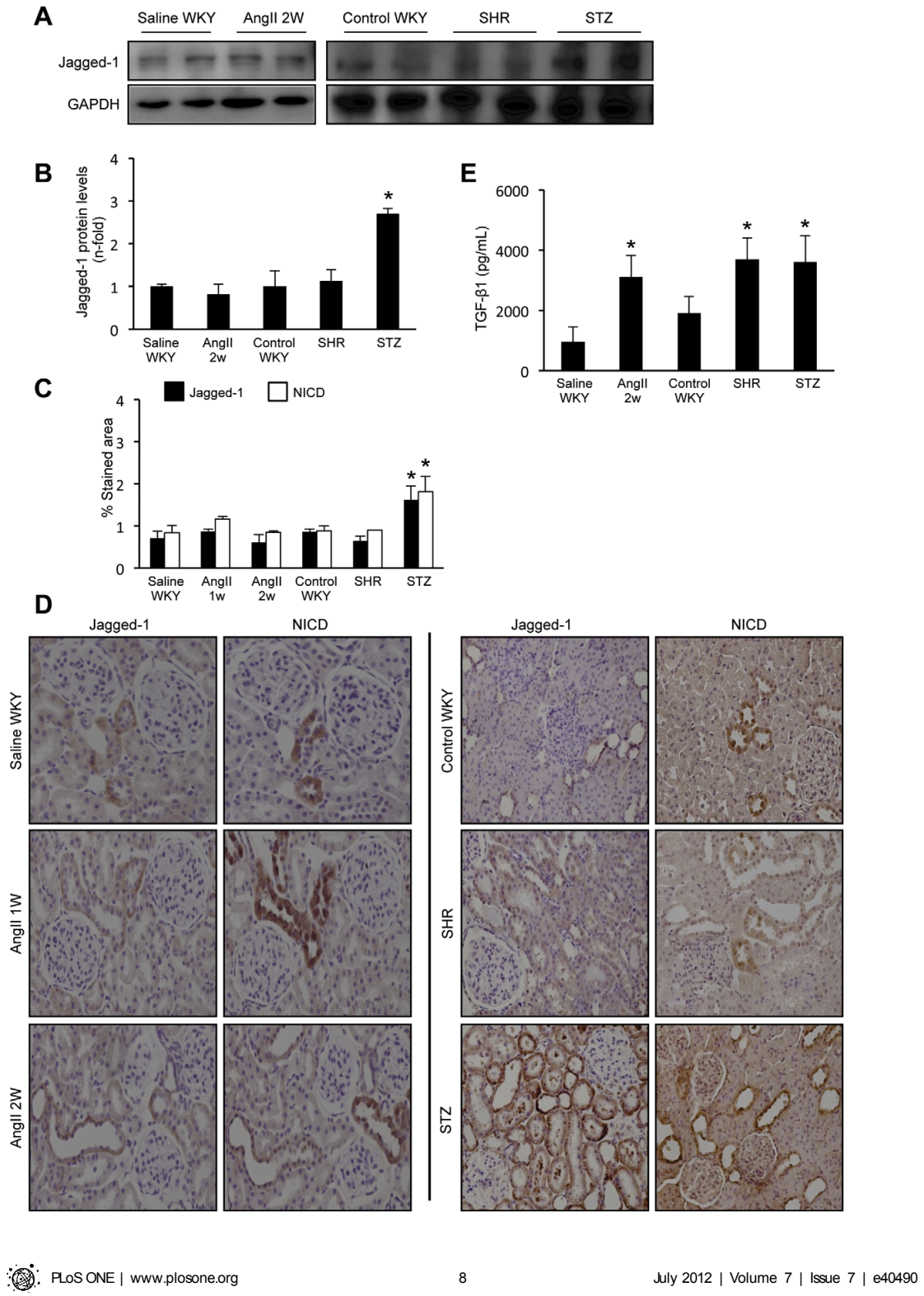
by local inhibition of chemokines and profibrotic factors, including TGF-b1, as described [38], but did not diminish renal Jagged-1 expression. Our in vitro studies clearly demonstrated that although TGF-b1 activated the Notch pathway in renal cells [5,18,27], AngII did not regulate this system. In cultured human tubular epithelial cells, we have found that AngII did not up-regulate Notch related genes, or increased Jagged-1 protein levels. Similar results were observed in podocytes and renal fibroblasts. These data clearly indicates that the Notch/ Jagged signaling system is not involved in renal damage associated to AngII and hypertension.

Many works have shown that the Notch/ Jagged signaling is essential for epithelial function and appears to contribute to EMT in embryogenesis and cancer [40,41]. In cultured tubular epithelial cells, suppression of the Notch pathway by pharmacological inhibition of c-secretase markedly inhibited phenotypic EMT changes induced by TGF-b1 [25,27]. In these cells TGF-b1 and AngII induce EMT by common mechanisms, including the Smad pathway and MAPK cascade [32,34]. Data presented here demonstrated that c-secretase inhibition did not modulate AngII-induced EMT, illustrating a different mechanism of action between AngII and TGF-b1. Interestingly, in these cells the Notch-1 ligand Jagged-1 induced a transition to a fibroblast-like phenotype and changes in EMT markers, such as loss of epithelial proteins and induction of mesenchymal markers, supporting the importance of Notch/ Jagged-1 activation in EMT regulation. However, the contribution of EMT to renal fibrogenesis is a matter of intense debate [24,41–44]. In this sense, in a transgenic mice model, the specific Notch activation in tubular and interstitial cells induced renal damage, characterized by increased cell proliferation of both cell types and fibrosis, but changes in EMT markers were not detected [20].

The relation between AngII and TGF-b1 in fibrosis is well known [28–32]. Many studies have demonstrated that TGF-b1 acts as a downstream mediator of AngII-induced renal fibrosis and both factors share several intracellular mechanisms involved in the regulation of ECM accumulation [28,29]. In tubular epithelial cells, we have demonstrated that although AngII increased active TGF-b1 levels, this endogenous TGF-b1 production is not enough to activate the Notch pathway. This observation support our in vivo findings in the models of AngII and hypertension-induced renal damage, both characterized by TGF-b1 overexpression and fibrosis in the absence of Notch pathway activation, as well as by the data in the model of UUO showing the lack of effect on renal Jagged-1 levels, but downregulation of TGF-b1 and renal damage, in response to AT1 antagonism. Interestingly, in a previous study the Notch blockade did not inhibit TGF-b1-induced upregulation of some profibrotic genes, such as CTGF, thrombospondin, MMP-9 [27] and, as described here, PAI-1. We have found that these genes are also upregulated by AngII independently of Notch activation. Importantly, CTGF has been described as a key downstream profibrotic mediator of AngII and TGF-b1 in several cells types, including renal cells [28]. PAI-1 has been involved in AngII-induced vascular fibrosis, independently of TGF-b [45,46]. These data indicated that several profibrotic-related events induced by TGF-b1 and AngII are independent of the Notch pathway activation.

Podocyte-specific Notch activation severely injures the glomerular filtration barrier in the kidney. Inhibition of the Notch pathway by podocyte-specific genetic ablation of the Notch coactivator RBP-Jk or pharmacological blockade of c-secretase reversed glomerular damage and re-established the filtration barrier [47]. Transgenic TGF-b1 overexpression cause podocyte injury, proteinuria and progressive glomerulosclerosis [48]. Moreover, Notch inhibition modulate TGF-b-mediated p53-dependent podocyte apoptosis [16]. In cultured human podocytes, TGF-b1, VEGF and high glucose activate Notch pahway and induce podocyte apoptosis [16,49]. However, in these cells AngII did not induce apoptosis [50], and did not increase Jagged-1 production. In a rat model of diabetic nephropathy pharmacological inhibition of the Notch signaling ameliorated proteinuria [49], showing that podocyte-specific Notch inhibition could be a good therapeutic option for proteinuric diseases, characterized by podocyte loss by apoptosis.

Divergent Notch functionality has been described depending on cell type. In the vasculature Notch-3 regulates vascular tone and cell growth/ apoptosis [51,52]. In these cells AngII inhibited Notch-3 [51], while in tubular epithelial cells, neither TGF-b1 nor AngII modulate Notch-3. In the kidney, Notch-3 upregulation was only observed in renal progenitors in human glomeruloesclerosis [14], supporting the role of Notch in renal regeneration.


A
Saline WKY
AngII 2W
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Jagged-1
GAPDH
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Jagged-1 protein levels (n-fold)
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TGF-β1 (pg/mL)
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Jagged-1
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AngII 1w
AngII 2w
D
Jagged-1
NICD
Saline WKY
AngII 1W
AngII 2W
Control WKY
SHR
STZ

Figure 6. Renal Notch pathway is not upregulated in AngII-infused rats or in SHR. Infusion of AngII (100 ng/kg/min) was done in normotensive rats from 24 hours to 2 weeks, saline infusion was used as control. Spontaneously hypertensive rats (SHR) of 16 weeks were studied, WKY of the same age were used as control, and streptozotocin-induced diabetic rats (STZ), a known model of activated renal Notch. Renal jagged-1 protein levels were elevated only in STZ rats, but not in AngII-infused or SHR rats. In total renal extracts, Jagged-1 levels were determined by western blot. Figure A shows a representative experiment of 2 animals per group and in B data as mean 6 SEM of 8 2 10 rats per group. *p, 0.05 vs control WKY. Jagged-1 and Notch intracellular domain (NICD) expression were evaluated by immunohistochemistry. C. Quantification of stained area as mean 6 SEM of 8–10 animals per group. *p, 0.05 vs control WKY. Figure D shows a representative picture of each group. Original magnification 200x. Renal TGF-b1 protein levels were determined by ELISA. Figure E shows data of active TGF-b1 in total renal extracts expressed as mean 6 SEM of 4–6 rats per group. *p, 0.05 vs its corresponding control group.
doi:10.1371/journal.pone.0040490.g006

Understanding the fine regulation of the Notch system in kidney injury is necessary since Notch signaling may impact kidney regeneration in addition to injury. In adult kidneys a resident renal cell population with progenitor activity strongly expresses members of the Notch signaling pathway [53]. In folic acid-induced renal injury, Notch inhibition did not modify acute renal injury and creatinine levels (a marker of renal recovery), but ameliorated renal lesions and fibrosis, observed at 7 days [20]. Interestingly, Notch activation was detected only in proliferating cells. In a model of acute tubular necrosis induced by ischemia-reperfusion, treatment with the Notch ligand Delta-like-4 facilitated renal recovery by increasing cell proliferation [21]. These data suggest that the described beneficial effects of Notch inhibition could be due to the modulation of cell proliferation. Furthermore, Notch activation in human renal progenitors stimulates cell proliferation, whereas its downregulation is required for differentiation toward the podocyte lineage. Indeed, persistent Notch activation induced podocyte death by mitotic catastrophe [14]. In mouse models of focal segmental glomerulosclerosis, Notch inhibition reduced podocyte loss and ameliorated proteinuria during the initial phases of glomerular injury, but Notch inhibition in the regenerative phases of glomerular injury reduced progenitor cell proliferation and worsened proteinuria and glomerulosclerosis [14].

There is a lack of effective therapy for chronic renal diseases. The beneficial effect of Notch inhibition in experimental proteinuric glomerular diseases, including diabetic nephropathy, shows the importance of Notch activation in podocyte failure. However, we describe here that the Notch pathway is not involved in AngII-induced fibrotic events. AngII contributes to renal damage progression, by inducing fibrosis-related events, and its blockade retards renal disease progression in humans. Although there are some current clinical trials using the c-secretase inhibitors for diseases as diverse as Alzheimer's and leukemia [47], our experimental studies does not support the potential beneficial effect of these drugs in AngII-mediate renal diseases. Our results show the complexity of the regulation of the Notch pathway in the kidney, and suggest that the involvement of this pathway in renal disease progression could be due to regulation of regeneration [14,21] rather than by its contribution to fibrosis.

Table 1. Data of the experimental models of spontaneously hypertensive rats (SHR) and diabetic nephropathy induced by streptozotocin injection (STZ).

	Systolic BP (mmHg)	*Protenuria* (mg/24h)	*Urinary Albumin* (mg/24h)
Control	112.06 3.9	4.56 1.8	0.446 0.3
SHR	143.16 10.2	8.86 3.6	2.006 0.7
STZ	121.26 4.8	8.66 3.6	2.316 0.4

doi:10.1371/journal.pone.0040490.t001

Our findings clearly indicate that more studies are necessary to improve the actual therapeutic approaches to limit renal damage progression, before the use of the c-secretase inhibitors for human diseases.

Methods

Ethics Statement

All experimental procedures were approved by the Animal Care and Use Committee of the IIS-Fundación Jimenez Diaz, according to the guidelines for ethical care of the European Community.

Experimental models

The model of systemic infusion of AngII was done in 3-month-old male Normotensive Wistar-Kyoto (WKY, Criffa, Barcelona, Spain). AngII (Biochem) dissolved in saline was infused at the dose of 100 ng/ kg/ min by subcutaneous osmotic minipumps (Alza Corp) for different time periods (from 24 hours to 2 weeks; n = 8 animals per group). A control group of saline-infused rats of the same age was also studied (n = 8 animals). SHR male rats of 16 weeks of age were studied as control group normotensive WKY of the same age were used (n = 8 animals per group).

Diabetic nephropathy (DN) was induced by two streptozotocin (STZ) injections (50 mg/ kg per day) or vehicle (0.01 mol/ L citrate buffer pH 4.5) in 6 week-old normotensive Wistar-Kyoto rats which were studied after 6 weeks of diabetes (n = 10 animals per group). Insulin (1–4 IU subcutaneous, Insulatard NPH) was administrated weekly to prevent death from 7 days after administration of STZ, once all animals had blood glucose levels . 400 mg/ dl. Systolic blood pressure was measured monthly in conscious, restrained rats by the tail-cuff sphygmomanometer (NARCO, Biosystems). The average of three separate measurements was calculated at each time point. Albuminuria in 24 hour/ urine samples was assessed by ELISA (Celltrend, Luckenwalde, Germany). The control group was the same as SHR rats.

The model of unilateral ureteral obstruction (UUO) was done in male C57BL/ 6 mice. The model was performed under isoflurane-induced anesthesia; the left ureter was ligated with silk (4/ 0) at two locations and cut between ligatures to prevent urinary tract infection (obstructed kidney), as described [38]. Some animals were treated with the AT1 antagonist Losartan (MSD, Spain; 10 mg/ kg per day; drinking water), starting 1 day before UUO and continued for 5 days (n = 6 mice per group).

At the time of sacrifice, animals were anesthetized with 5 mg/ kg xylazine (Rompun, Bayer AG) and 35 mg/ kg ketamine (Ketolar, Fisher) and the kidneys perfused in situ with cold saline before removal. A piece of the kidney (2/ 3) was fixed, embedded in paraffin, and used for immunohistochemistry, and the rest was snap-frozen in liquid nitrogen for renal cortex RNA and protein studies. In UUO model, studies were done comparing both kidneys (contralateral and obstructed) in each mouse. In addition, a control group of sham-operated mice was also done, showing the same results than contralateral kidneys (data not shown).

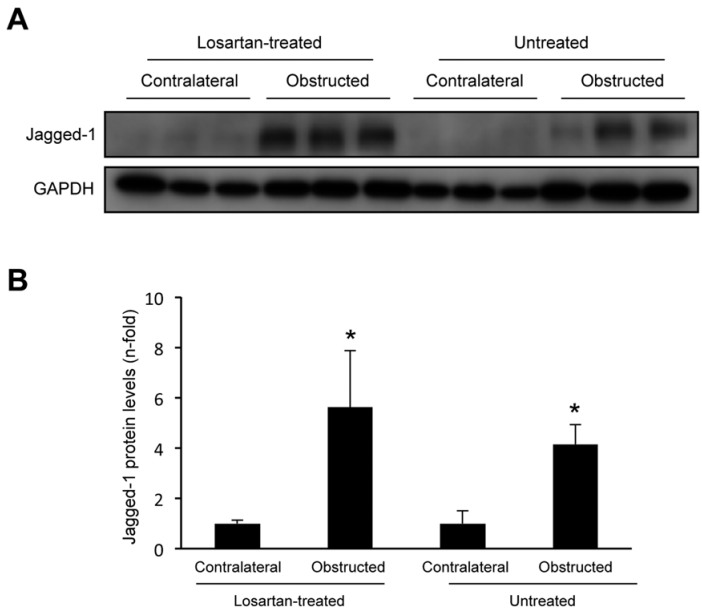

Figure 7. AT1 antagonism increased renal Jagged-1 protein levels in the model of unilateral ureteral obstructed kidneys in mice. The figure A shows a representative experiment of Jagged-1 protein levels evaluated by western blot and in B data as mean 6 SEM of 6 animals per group. *p, 0.05 vs contralateral kidneys.
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Cell cultured studies

Human renal proximal tubular epithelial cells (HK-2 cell line, ATCC CRL-2190) were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), 2 mmol/ L glutamine, 100 U/ mL penicillin, 100 mg/ mL streptomycin, 5 mg/ mL Insulin Transferrin Selenium (ITS) and 36 ng/ mL hydrocortisone in 5% CO_2 at 37uC. At 60–70% of confluence, cells were growth-arrested in serum-free medium for 24 hours before the experiments.

Human podocytes are an immortalized cell line, transfected with a temperature-sensitive SV40 gene construct and a gene encoding the catalytic domain of human telomerase [54]. At a permissive temperature of 33uC, the cells remain in an undifferentiated proliferative state, whereas raising the temperature to 37uC results in growth arrest and differentiation to the parental podocyte phenotype. Undifferentiated podocyte cultures were maintained at 33uC in RPMI 1640 medium with penicillin; streptomycin; insulin, transferrin, and selenite; and 10% FBS. Once cells had reached 70 to 80% confluence, they were cultured at 37uC for at least 14 days before use, when full differentiation had taken place. For experiments, cells were cultured in serum-free medium 24 hours before the addition of the stimuli and throughout the experiment.

Murine renal cortical fibroblasts (TFB cell line) originally obtained from Dr. Eric Neilson (Vanderbilt University) were grown in RPMI 1640 with 10% FBS, 2 mM glutamine, 100 U/ ml penicillin and 100 mg/ ml streptomycin in 5% CO_2 at 37uC [55]. At 60–70% of confluence, cells were growth-arrested in serum-free medium for 24 hours before the experiments.

Cells were cultured in six-well plates, serum starved for 24 hours and treated with vehicle (PBS), recombinant human TGF-b1 (Peprotech), recombinant Ang II (Sigma) or recombinant human Jagged-1 (R&D systems) for 24 or 48 hours in serum-free medium. The c-secretase inhibitor IX (DAPT, Calbiochem) was added together with TGF-b1 at 36 10^{-8} mol/ L DAPT for 24 hours. DMSO, used as solvent, had no effect on cell viability and gene expression (not shown). Cells were used for protein or RNA studies, and the supernatants (cell-conditioned media) for TGF-b1 measurements.

Protein studies

Cells were homogenized in lysis buffer (50 mmol/ L Tris/ HCl; 150 mol/ L NaCl; 2 mmol/ L EDTA; 2 mmol/ L EGTA; 0.2% Triton X-100; 0.3% IGEPAL, 10 ml/ mL protease inhibitors cocktail; 1 ml/ mL PMSF, 1 ml/ mL and 10 ml/ mL orthovanadate) and then separated by SDS-polyacrylamide gel electrophoresis. Jagged-1 and EMT markers were determined in total protein extracts by western blot, 20 mg of proteins were loaded in each lane. Protein content was determined by the BCA method (Pierce, Rockford). The efficacy of protein transfer to the membranes was assessed by Red Ponceau staining (data not shown). To evaluate equal loading, membranes were stained with anti-GAPDH antibody. The autoradiographs were scanned using the GS-800 Calibrated Densitometer (Quantity One, Bio-Rad). The following primary antibodies were employed [dilution]: Jagged-1 (Santa Cruz, [1:500]); Vimentin (R&D, [1/ 10000]); pan-Cytokeratin (Sigma-Aldrich, [1/ 10000]); GAPDH (Chemicon International, [1/ 5000]).

Paraffin-embedded kidney biopsy specimens were used for evaluation of Jagged-1 and Notch intracellular domain (NICD) staining. Specific biotinylated secondary antibodies were used, followed by streptavidin–horseradish peroxidase conjugate, and developed with diaminobenzidine. The following primary antibodies were employed [dilution]: Jagged-1 (Santa Cruz, [1:100]); NICD (Abcam, [1:300]). Briefly, 5 mm thick renal sections were

deparaffinized and endogenous peroxidase was blocked by 3% H_2O_2 for 20 min. Then, the sections were incubated overnight at 4uC with specific primary antibodies. The specificity was checked by omission of primary antibodies.

For immunocytochemistry experiments, cells were grown on coverslips. After incubation, cells were fixed in paraformaldehyde 4% and permeabilized with 0.2% Triton-X100 for 10 min. After blocking with 3% BSA, they were incubated with primary antibodies: anti NICD (abcam, 1:300) overnight at 4uC, followed by a AlexaFluorH 488 secondary antibody (Invitrogen) for 1 h. Nuclei were stained with 49,6-Diamidino-2-phenyindole (DAPI). Absence of primary antibody was used as negative control. Samples were mounted in Mowiol 40–88 (Sigma-Aldrich) and examined by a Leica DM-IRB confocal microscope.

For the evaluation of TGF-b1 protein levels an ELISA kit from eBioscience was used, and TGF-b1 levels were quantified by comparison with a standard curve. In the in vitro studies, the conditioned media were collected to evaluate active TGF-b1 (as described above), and data were expressed as fold-change over untreated cells. In the different experimental models, renal TGF-b1 protein levels were evaluated in 0.1 mg/mL of total renal protein extracts, and data were expressed as fold-change the mean of value of the corresponding control animal in each model.

Gene expression studies

Total RNA was isolated from cells with Trizol (Invitrogen). cDNA was synthesized from 2 mg of total RNA primed with random hexamer primers using the High capacity cDNA Archive Kit (Applied). Multiplex real time PCR was performed using Applied Biosystems expression assays (Taqman Fam fluorophore) as follows: Jagged1: Hs01070032_m1; Notch1: Hs 00413187_m1; Delta1: Hs01128541_m1, Notch3: Hs00194509_m1, Vimentin: Hs00185584_m1; MMP-9: Hs00234579_m1 PAI-1: Hs00167155_m1 and CTGF: Hs00170014_m1. Data were normalized to 18S eukaryotic ribosomal RNA: 4210893E (Vic). The mRNA copy numbers were calculated for each sample by the instrument software using Ct value ("arithmetic fit point analysis for the lightcycler"). Results were expressed in copy numbers, calculated relative to unstimulated cells after normalization against 18S.

Statistical analysis

Results are expressed as n-fold increase over control as mean 6 SEM. Differences between groups were assessed by Mann-Whitney test. p, 0.05 was considered significant. Statistical analysis was conducted using the SPSS statistical software (version 11.0, Chicago, IL).

Supporting Information

Figure S1 AT1 antagonist treatment ameliorates renal damage in the model of unilateral ureteral obstruction in mice. Animals were treated daily with the AT1 antagonist losartan, starting 1 day before unilateral obstruction, and animals were studied 5 days after obstruction. A. In obstructed kidneys there is a marked inflammatory cell infiltration that was diminished in Losartan-treated mice. The figure A shows of CD3 lymphocytes immunostaining of a representative animal of each group (magnification 200x). B. Losartan downregulated proinflammatory factors. The MCP-1 gene expression was evaluated by real time PCR. C. Losarta-n diminished renal TGF-b1 protein levels. TGF-b1 was determined by ELISA. Data is shown as mean 6 SEM of 6 animals per group. *p, 0.05 vs contralateral kidneys. # p, 0.05 vs untreated.
(TIF)

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Author Contributions

Conceived and designed the experiments: CL RRD AO MRO. Performed the experiments: CL RRD ABM SRM RRRD MA. Analyzed the data: CL RRD ABM MA MRO. Contributed reagents/ materials/ analysis tools: AO JE SM MRO. Wrote the paper: CL JE SM MRO.

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5. Estudio traslacional de la vía Notch en Nefropatía Hipertensiva

El papel del sistema Notch-Jagged en la patología renal no está bien esclarecido. Se ha descrito una activación local del sistema en una gran variedad de nefropatías crónicas progresivas humanas, incluida la nefropatía diabética, que se correlaciona con la pérdida de funcionalidad renal.[158] Sin embargo, su implicación en la fibrosis *in vivo* no ha sido demostrada. El efecto beneficioso de la modulación de la vía Notch en la progresión de la enfermedad renal es todavía controvertido.[13,67,167] En esta tesis hemos descrito que la activación de la ruta Notch no está implicada en la fibrosis renal experimental inducida por Ang II o por hipertensión. El objetivo de este trabajo fue evaluar si la vía Notch está activada en la fibrosis renal asociada a nefroesclerosis hipertensiva en humanos en comparación con otras nefropatías progresivas

En pacientes con nefroesclerosis hipertensiva se observó baja expresión renal de Jagged-1 (principal ligando de la vía) y de Notch-1 activado, no existiendo asociación entre la fibrosis túbulo-intersticial y los niveles de expresión renal de estas proteínas. Por el contrario, en las patologías glomerulares estudiadas (nefropatía membranosa no progresiva y progresiva y nefropatía diabética) se observó una elevada expresión de los transcritos Jagged-1, HES-1 y TGF-β, y de las proteínas Jagged-1 y Notch-1 activo, localizado principalmente en células túbulo-epiteliales. Los niveles de expresión de los componentes de la vía Notch se relacionaron con el grado de fibrosis túbulo-intersticial, lo que confirma la activación de esta vía en nefropatías progresivas.

En este trabajo hemos demostrado que la vía Notch no está activada en el riñón de pacientes con nefroesclerosis hipertensiva, ampliando los resultados observados en los modelos experimentales de ratas espontáneamente hipertensas y de daño por administración sistémica de Ang II a la patología humana.

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Translational study of the Notch pathway in hypertensive nephropathy

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ABSTRACT

Introduction: The Notch signalling pathway is activated in a wide variety of human renal diseases. We have recently demonstrated that the activation of this pathway is not involved in experimental renal fibrosis induced by angiotensin II or hypertension. **Objectives:** To assess whether the Notch pathway is activated in renal fibrosis related to hypertensive nephrosclerosis. To test the hypothesis, various glomerular diseases characterised by tubulointerstitial fibrosis were analysed. **Method:** Renal biopsies were performed on patients with hypertensive nephrosclerosis, in comparison with diabetic nephropathy and membranous nephropathy at various stages. Gene and protein expression were evaluated by in-situ hybridisation and immunohistochemistry respectively. **Results:** In hypertensive nephrosclerosis low renal expression of notch-related proteins was observed. There was no link between tubulointerstitial fibrosis and the levels of these proteins. By contrast, in the glomerular diseases studied we observed high expression of the transcripts Jagged-1, HES-1 and TGF-β and the proteins Jagged-1 y Notch-1, localised primarily in tubuloepithelial cells. The levels of expression of the components of the Notch pathway correlate to the degree of tubulointerstitial fibrosis, which confirms the activation of this pathway in progressive nephropathies. **Conclusions:** Our data demonstrate that the Notch pathway is not activated in the kidneys of patients with hypertensive nephropathy, which extends the results of experimental models of kidney damage related to hypertension to the realm of human pathology. Our studies provide new information on the complex regulation of the Notch pathway in the kidney.

Keywords: Hypertension. Renal damage. Angiotensin. Notch. fibrosis.

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Estudio traslacional de la vía Notch en nefropatía hipertensiva

RESUMEN

Introducción: *La ruta de señalización de Notch está activada en una gran variedad de patologías renales humanas. Recientemente hemos demostrado que la activación de esta ruta no estaría implicada en la fibrosis renal experimental inducida por angiotensina II o hipertensión.* ***Objetivos:*** *Evaluar si la vía Notch está activada en la fibrosis renal asociada a nefroesclerosis hipertensiva. Para validar la hipótesis se estudiaron varias patologías glomerulares caracterizadas por fibrosis túbulo-intersticial.* ***Métodos:*** *Se utilizaron biopsias renales de pacientes con nefroesclerosis hipertensiva, en comparación con nefropatía diabética y nefropatía membranosa en diferentes etapas de progresión. La expresión génica y proteica se evaluó por hibridación in situ e inmunohistoquímica, respectivamente.* ***Resultados:*** *En nefroesclerosis hipertensiva se observó baja expresión renal de proteínas de la vía Notch, no existiendo asociación entre la fibrosis túbulo-intersticial y los niveles de estas proteínas. Por el contrario, en las patologías glomerulares estudiadas se observó una elevada expresión de los transcritos Jagged-1, HES-1 y TGF-β, y de las proteínas Jagged-1 y Notch-1, localizados principalmente en células túbulo-epiteliales. Los niveles de expresión de los componentes de la vía Notch se relacionaron con el grado de fibrosis túbulo-intersticial, lo que confirma la activación de esta vía en nefropatías progresivas.* ***Conclusiones:*** *Nuestros datos muestran que la vía Notch no está activada en el riñón de pacientes con nefropatía hipertensiva, ampliando los resultados de los modelos experimentales de daño renal asociado a hipertensión a la patología humana. Nuestros estudios aportan nueva información sobre la compleja regulación del sistema Notch en el riñón.*

Palabras clave: *Hipertensión. Daño renal. Angiotensina. Notch. fibrosis.*

INTRODUCTION

The Notch signalling pathway is involved in cellular proliferation, differentiation and apoptosis processes[1,2].

The members of this pathway include Notch receptors 1/2/3/4, which have two subunits not covalently linked, and the Jagged 1,2 and delta-like 1,3,4 ligands[3]. The Notch pathway is activated following the interaction of the Notch receptor and its binder, which causes the proteolytic cut of the transmembrane section of the Notch receptor, via the γ-secretase enzyme. This release the intracellular section of the receptor, called the Notch intracellular domain (NICD). This active domain migrates to the nucleus, binds to the RBP-Jκ transcription factor (recombination signal-binding protein 1 for J-kappa) and activates the target genes, including HES (hairy/enhancer of split) and HERP (HES-related transcription factor-1), which are transcriptional repressors that act as negative effectors of the pathway[1].

The Notch pathway is used by multicellular organisms to specify cell fate during the formation of complex structures, including the kidney[4], and participates in physiological and pathological processes, such as cancer[5], the function and regeneration of the vasculature[1], endothelial cell[6] differentiation and angiogenesis[7]. In the kidney, Notch pathway activation in podocytes and renal progenitor cells associated with pathological processes in glomerulonephritis has been described[8]. In addition, experimental model studies show that the over-expression of Notch in podocytes is associated with albuminuria and glomerusclerosis[9,10]. In renal biopsies of patients with diabetic nephropathy (ND) we have described an increase in Jagged-1 and HES-1 gene expression[11]. In an extensive study by Suztak et al., an increase in various components of the Notch pathway was observed in progressive nephropathies[12]. However, the beneficial effect of modulation of the Notch pathway in kidney disease progression remains controversial[9,13,14].

We have recently demonstrated that the Notch pathway activation is not involved in experimental renal fibrosis induced by angiotensin II (Ang II) or hypertension[15]. Our objective has been to try to transfer the results obtained in experimental models to the realm of human pathology, evaluating whether the Notch pathway is activated in patients with hypertensive nephropathy (HN), comparing them with progressive nephropathies exhibiting different degrees of tubulointerstitial fibrosis.

MATERIAL AND METHOD

Patient samples

The study was based on the retrospective analysis of renal biopsies studied in the Nephrology Department of the Instituto de Medicina de la Universidad Austral de Chile, Valdivia (Chile). The renal biopsies obtained by percutaneous puncture were carried out as a diagnostic and/or prognosis procedure, and with the patient's informed consent. There were no significant differences in the most relevant clinical data between the HN group and the ND and membranous nephropathy (MN) groups. The concrete patient data from whom the clinical information was taken is as follows: HN: 4 males/6 females; average age: 49.2 years; estimated glomerular filtration rate (eGFR): < 60ml/min/1.73m^2 = 4/8; tubulointerstitial fibrosis score 1. Patient data DN + MN = 15 males/9 females; average age: 53.5 years; eGFR <60ml/min/1.73m^2 = 11/19, tubulointerstitial fibrosis score 1.69. With respect to pharmacological treatment, the majority of patients in all the groups studied were receiving angiotensin-converting enzyme inhibitors (ACE inhibitors).

The renal biopsies are from patients with histopathological diagnosis of HN (n = 10), with DN (n = 8) and with MN. These cases were divided into patients with non-progressive MN (np-MN; n = 8, absent or slight tubulointerstitial fibrosis) and with progressive MN (p-MN; n = 8, moderate or severe tubulointerstitial fibrosis). As control tissues, biopsies from patients with minimal changes disease (MCD; n = 5) were used, pathology which does not present tubulointerstitial fibrosis. Interstitial fibrosis, defined by Masson's trichrome technique (which detects the presence of interstitial collagen) and the tubulointerstitial cellular infiltration were classified into four groups, according to the extension and presence of atrophy and tubular degeneration: (0) normal, (1) affecting up to 25 % of the cortex, (2) affecting 25 % to 50 % of the cortex, and (3) extensive damage to more than 50 % of the cortex, as described previously in other studies[16]. All the biopsies were evaluated by two pathologists, whose readings coincided, without prior knowledge of other studies carried out on these biopsies.

The renal tissue obtained was fixed in formalin 4 % in phosphate buffer and/or Bouin solution. It was then dehydrated and embedded in paraffin following the conventional histological technique. For the development of the techniques described, a series of cuts of 5 µm thickness were made, which were mounted on microscope slides previously treated with 2 % aminopropiltrietoxisilane.

In-situ hybridisation

In order to evaluate the levels of gene expression, *in-situ* hybridisation, as previously described, was used[17]. The hybridisation reaction was carried out overnight at 37 ºC using biotin-stained probes (200ng/ml). The detection was made with avadin-alkaline phosphatase conjugate, using NBT/BCIP (nitro bluetetrazolium/5-bromo-4-chloro-3-indolyl phosphate) as the enzyme substrate. The reaction specificity was confirmed by RNase treatment (100mg/ml) or by absence of the probe.

Probes used for Jagged-1 detection (Sigma):5'-C C T G A C A G T A T T A T T G A A A A G G C T-3',

5'-GTACGGCTGGCAAGGCTTGTACTG-3', 5'-CACGCCTGCCTCTCTGATCCCTGT-3'.

Probes used for HES-1 detection (Sigma): 5'-CTTCTCTCCTTGGTCCTGGAACAG-3', 5'-AGCTCGCGGCATTCCAAGCTGGAG-3', 5'-CTGCGCTGAGCACAGACCCAAGTG-3'.

Probe used for TGF-β detection (MaxinBiotechInc): Biotinylated probe of 163pb, made by PCR using a human cDNA and biotinylated dNTPs.

Immunohistochemistry

Immunohistochemistry was used to evaluate the levels of protein expression, using the following primary antibodies: anti-Jagged-1 (1:50, Santa Cruz), active anti-Notch-1 (1:300, Abcam) and anti-α-SMA (1:50, Dako). For this, endogenous peroxidase was blocked in renal sections using 3 % H_2O_2 for 20 minutes. The tissue was treated in microwaves with a citrate buffer solution 0.1mM 6.0 pH at 94 ºC for 10 minutes. Secondary biotinylated antibodies were used, followed by HRP-conjugate and revealed with diaminobenzidine. The reaction specificity was determined by primary antibody omission or the use of a non-immune serum.

Statistical analysis

Statistical analysis was carried out using GraphPadInstant software (GraphPadSoftware, San Diego, CA). The evaluation of the intensity and distribution of the staining for immunohistochemistry and *in-situ* hybridisation was determined using computerised analysis of images, with no knowledge of the group that the sample belonged to, and expressed as mean ± SEM. The Mann-Whitney test was used to determine significant differences between the analysed groups. A *P* value of <.05 was considered to be statistically significant. The Spearman correlation was used to relate Jagged-1, HES-1 and TGF-β expression and tubulointerstitial fibrosis. A *P* value of <.01 was considered to be statistically significant.

RESULTS

The Notch pathway in hypertensive nephrosclerosis

The presence of the Notch ligand, Jagged-1, in patients with HN was evaluated using immunohistochemistry. Low levels of Jagged-1 expression were observed in those patients (Figure 1A). For the validation of the results and the technique used, we studied, in parallel, patient samples with DN. np-MN and p-MN, where renal activation of the Notch pathway was previously described[13]. In these samples, a significant increase in Jagged-1 expression at the cytoplasmic level of tubular cells was observed (Figure 1A). It is worth noting that in patients with MCD, positive staining of Jagged-1 was barely observed, presenting values similar to those of HN (Figure 1B).

NICD was released following the binding of the ligand (Jagged-1) to the Notch receptor, which translocated to the nucleus and acted as a transcription factor.

Previous studies have demonstrated that Notch pathway activation is associated with the progression of kidney disease in a large number of disorders, including DN[11,12], but there is no clear data on HN. Therefore, we studied the possible correlation between renal fibrosis and Notch pathway activation, analysing serial biopsies from the patient group. In the HN samples, no correlation was found between the degree of tubulointerstitial fibrosis and the intensity of Jagged-1 protein expression (Figure 3A), presenting Jagged-1 values similar to MCD, nephropathy that was not associated with tubulointerstitial fibrosis. By contrast, there was a significant positive correlation on the evaluation of the rest of the study samples (Figure 3B). These results suggest that the activation of the Notch Pathway in HN is not related with the progression of the disease.

The Notch pathway is activated in progressive membranous nephropathy associated with fibrosis

In order to determine the relationship between the Notch pathway and fibrosis in chronic kidney disease and building on previous studies[11,12], we evaluated the gene expression of Jagged-1 and HES-1 in patients with p-MN and np-MN, and compared their expression with the control tissue. In the np-MN and p-MN samples there was a marked induction of mRNA Jagged-1 and mRNA HES-1 at the tubular level. In contrast, in patients with MCD, used as controls as they did not present fibrosis, their expression was low or absent (Figure 4A).

Experimental studies have shown that TGF-β is a key growth factor in renal fibrosis. In patients with progressive kidney disease we have previously described that the TGF-β transcript expression is increased[18]. In Figure 4A, the co-localisation of mRNA of Jagged-1, HES-1 and TGF-β is observed in all the analysed MN cases. Progressive cases show a greater increase in mRNA expression of Jagged-1, HES-1 and TGF-β, compared with non-progressive cases (Figure 4B). In addition, a significant positive correlation between the degree of tubulointerstitial fibrosis and each transcript analysed was found (Figure 4C). We also evaluated the expression of the mesenchymal marker α-smooth muscle actin (α-SMA) and the presence of interstitial collagen in the tissue samples. In the α-SMA control tissue, it was only present in arterioles and there was no evidence of collagen. In np-MN, an increase in

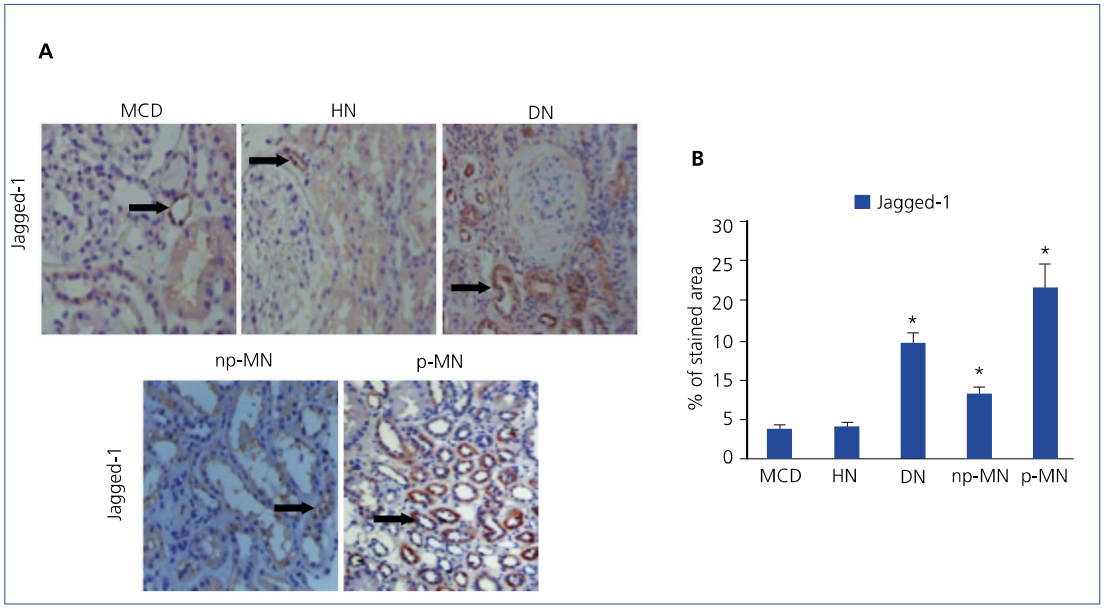

Figure 1. Immunodetection of Jagged-1 in biopsies of patients with minimal glomerular lesion (n = 5), hypertensive nephrosclerosis (n = 10), diabetic nephropathy (n = 8), and non-progressive (n = 8) and progressive (n = 8) membranous nephropathy.

A) Shows representative sections of human biopsies from all the cases analysed. B) Results are expressed as % of the stained area, as mean ± SEM (*P<0.01 vs. MCD).

MCD: minimal change disease; DN: diabetic nephropathy; HN: hypertensive nephrosclerosis; np-MN: non-progressive membranous nephropathy; p-MN: progressive membranous nephropathy.

α-SMA expression and collagen in the tubulointerstitial area was observed, and with greater extension in p-MN (Figure 4A). Our findings from renal biopsies of patients with p-MN and np-MN show a relation between Jagged-1 induction and tubulointerstitial damage. This suggests that Jagged-1 could participate in fibrosis regulation in the kidney.

DISCUSSION

From biopsies of HN patients, with different degrees of tubulointerstitial fibrosis, we have shown that the Notch pathway is not activated in the kidney. This builds on and confirms our previous *in vitro* studies and studies in animal models of hypertension and damage mediated by Ang II[15], transferring to human pathology the hypothesis that Ang II does not regulate the Notch pathway in the adult kidney.

Ang II actively participates in the progression of kidney damage, as it is able to regulate various pathological processes, such as inflammation and fibrosis. Drugs that inhibit Ang II, ACE inhibitors and Ang II receptor antagonists, are one of the best therapeutic strategies for the treatment of progressive kidney diseases. This is due to their actions beyond the control of blood pressure, providing end-organs protective effects[18]. Recently, we have described how the Notch pathway is not activated in response to Ang II in renal cell cultures, including podocytes, epithelial tubular cells and fibroblasts. This demonstrates a key difference between the mechanisms activated by TGF-β and Ang II in the kidney[15]. Previous studies suggest functional interactions between TGF-β and the Notch signalling pathway[19]. Both signalling pathways are important for cellular differentiation control during development and Jagged-1 expression has been described as dependent on TGF-β in epithelial cells[20,21]. Various studies have demonstrated that the Notch pathway is essential for epithelial function and also contributes in epithelial-mesenchymal transition (EMT) in embryogenesis and cancer[22,23]. Studies on tubuloepithelial cell cultures have shown that the pharmacological blocking of this pathway, inhibiting the γ-secretase enzyme, restored the changes

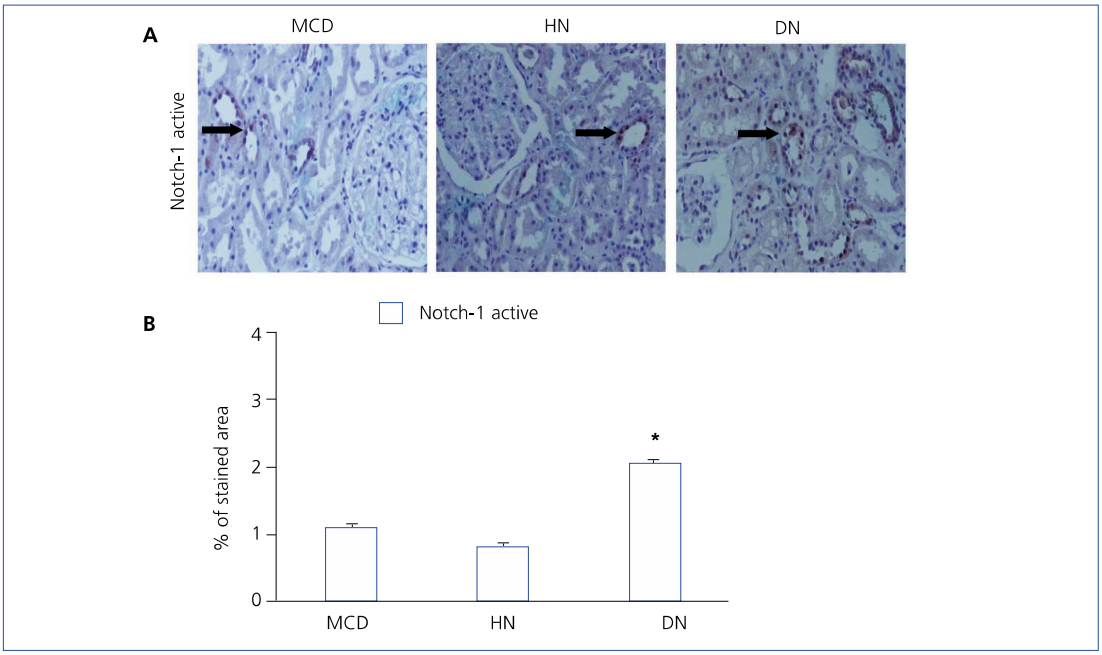

Figure 2. Immunodetection of activated Notch-1 in biopsies of patients with minimal glomerular lesion, hypertensive nephrosclerosis and diabetic nephropathy.
A) Shows representative sections of the cases analysed. B) Results are expressed as % of the stained area, as mean ± SEM (*P<0.01 vs. MCD).
MCD: minimal glomerular lesion; DN: diabetic nephropathy; HN: hypertensive nephrosclerosis.

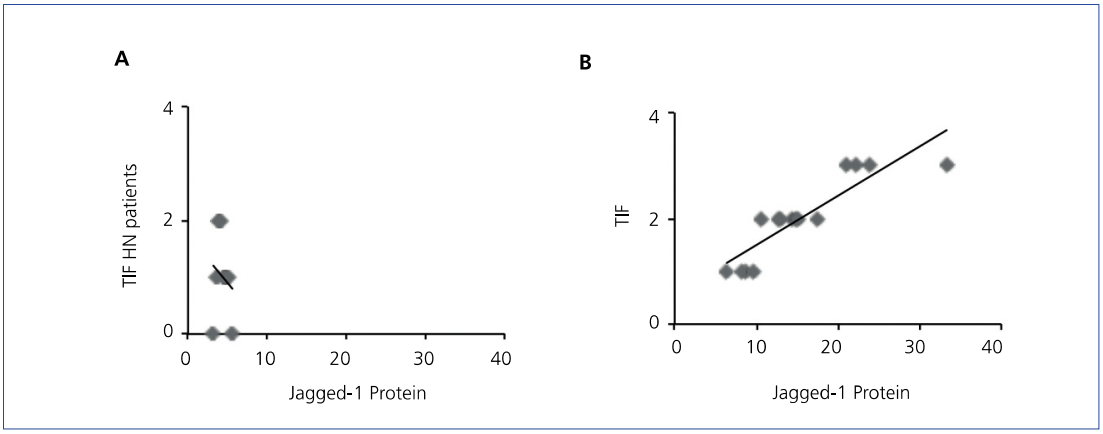

Figure 3. Activation of the Notch pathway is not related to renal fibrosis in hypertensive nephrosclerosis.
A) In patients with hypertensive nephrosclerosis there is no correlation between the degree of tubulointerstitial fibrosis and the level of Jagged-1 protein expression (r = 0.0307, n = 10 patients). B) Significant positive correlation between the degree of tubulointerstitial fibrosis and Jagged-1 in the rest of the pathologies studied, including diabetic nephropathy, progressive and non-progressive membranous nephropathy (r = 0.784; P<0.01; n = 8 patients per group).
TIF: tubulointerstitial fibrosis; HN: hypertensive nephrosclerosis.

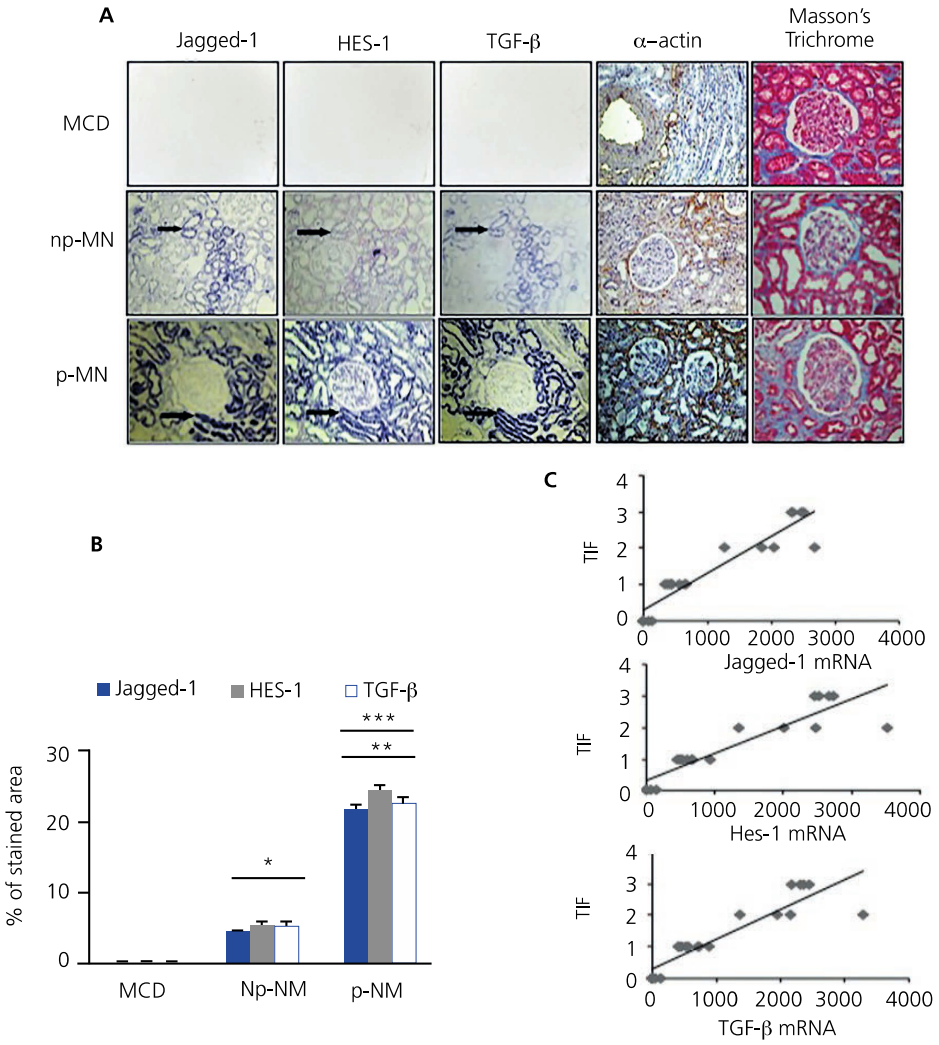

Figure 4. Activation of the Notch pathway is related to renal fibrosis in membranous nephropathy.
A) Levels of gene expression of the transcribed Jagged-1, HES-1 and TGF-β in minimal glomerular lesion, and progressive and non-progressive membranous nephropathy. The genes were detected by in-situ hybridisation using specific probes in serial sections of renal tissue. Co-localisation of Jagged-1 mRNA, HES-1 and TGF-β in renal tissue. The arrows indicate positive signal. Staining for α-actin and Masson's trichrome is also observed. B) Densitometric quantification of the transcripts. Results are expressed as % of the stained area. Results are shown as mean ± SEM of the analysed cases (n = 5 of MCD, n = 8 rest of groups) (*P<0.01 compared to MCD; **P<0.01 vs. MCD; ***P<0.01 vs. np-MN). C) The figures show a significant positive correlation between the degree of tubulointerstitial fibrosis and the intensity of the reactive point of the transcribed: Jagged-1 (r = 0.92, P<.01), HES-1 (r = 0.87, P<0.01) and TGF-β (r = 0.92, P<0.01), determined by Spearman correlation.
TIF: tubulointerstitial fibrosis; MCD: minimal change disease; np-MN: non-progressive membranous nephropathy; p-MN: progressive membranous nephropathy.

in EMT markers induced by TGF-β[20,24]. However, it does not modulate the ETM caused by Ang II[15]. The contribution of ETM in renal fibrogenesis is intensely debated[24]. Consequently, the renal over-expression of Notch in kidney damage models did not modulate ETM markers, although it caused tubulointerstitial fibrosis[13]. In this study, we have demonstrated that the Notch pathway is not activated in the kidney of HN patients, extending the result observed in experimental models of hypertension and AngII-induced renal damage to the human hypertensive disease.

Various experimental studies have observed that the use of pharmacological inhibitors or soluble Notch ligands mitigates kidney failure[9,25]. In addition, studies on transgenic mice with Notch deletion at the podocyte level have demonstrated the involvement of this pathway in tubulointerstitial fibrosis [13]. Experiments carried out on patient samples with p-MN show that Jagged-1 and HES-1 activation are linked with the degree of tubulointerstitial fibrosis and with the increase of TFG-β. This confirms the earlier extensive study that showed Jagged-1, Jagged-2 and Notch-1 activation in progressive human nephropathies[12], as well as the previous results of our group in DN[11].

Overall, our results show the complex regulation of the Notch pathway in the kidney, confirming the association between Notch pathway activation and renal fibrosis in progressive human pathologies. Currently there are clinical studies using γ-secretase inhibitors in a range of diseases, such as Alzheimer's disease and leukaemia. As a result, it could also be a new therapeutic target for chronic kidney disease[25]. However, the fact that Ang II does not regulate this pathway and that it is not activated in patients with hypertension demonstrates the complexity of the Notch system regulation in the adult kidney.

Conflicts of interest

The authors declare that they have no conflicts of interest related to the contents of this article.

Acknowledgements

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IV. DISCUSIÓN

El principal hallazgo de esta tesis es que Gremlin se une al receptor VEGFR2 en el riñón y en células renales en cultivo, y activa esta vía de señalización induciendo diversas acciones, incluidas la regulación de factores pro-inflamatorios y pro-fibróticos, la inducción de una respuesta inflamatoria renal y la activación de diversos mecanismos moleculares, como son la ruta del NF-kB y la vía de señalización Notch.

1. Gremlin se une al receptor VEGFR2 y activa esta vía de señalización en células renales e *in vivo* en el riñón

Estudios previos, mediante análisis de resonancia de plasmón superficial, han demostrado la habilidad de Gremlin para unirse al dominio extracelular del receptor VEGFR2 en células endoteliales y participar en la respuesta angiogénica.[150] Nuestros estudios *in vitro* muestran que Gremlin se une a VEGFR2 en células túbulo-epiteliales en cultivo, como lo demostramos por estudios de inmunoprecipitación y formación de complejos Gremlin/VEGFR2 y por microscopía confocal en tiempo real, que permite visualizar la unión directa de Gremlin a la célula, efecto que fue inhibido mediante silenciamiento génico del receptor VEGFR2.

VEGFR2 es un receptor tirosina quinasa, que es activado por diferentes miembros de la familia de VEGF. En humanos se han descrito 3 ligandos canónicos para este receptor: VEGFA, VEGFC y VEGFD.[192,222] La fosforilación de diferentes residuos de tirosinas se produce tras la unión del ligando a VEGFR2, activando diferentes respuestas celulares.[171,176,192] La unión de Gremlin al receptor VEGFR2 difiere de los ligandos canónicos de este receptor ya que Gremlin no interacciona con neuropilina-1.[33] Sin embargo, las diferencias en señalización y respuestas fisiológicas no se conocen bien.

Nuestros estudios *in vivo* demuestran que la administración de Gremlin recombinante en riñón normal sano, induce una rápida y sostenida activación de la fosforilación de VEGFR2, principalmente localizado en células túbulo-epiteliales proximales, que son las células donde mayoritariamente se une Gremlin *in vivo*, como hemos observado al inyectar la proteína marcada con el fluoróforo Cy5. En estas células, tanto *in vivo* como *in vitro*, Gremlin induce la fosforilación de VEGFR2 en la Tyr951 y Tyr996. Estos datos sugieren que el receptor VEGFR2 es un receptor funcional de Gremlin en riñón.

2. Gremlin a través de VEGFR2 contribuye a la regulación de la respuesta inflamatoria renal

Gremlin afecta distintos procesos durante el crecimiento, la diferenciación y el desarrollo, mediante su heterodimerización con BMPs, impidiendo que estos se puedan unir a sus receptores.[79,141,148] En contraste, muchos estudios *in vitro* demuestran efectos de Gremlin independientes de BMPs que participa en la regulación de diversos procesos, incluidos angiogénesis, migración, fibrosis y activación de varias señales intracelulares.[30,31,150,191,225]

Estudios anteriores han demostrado claramente que VEGF/VEGFR2 está estrechamente vinculado a la angiogénesis, tanto en procesos fisiológicos como patológicos.[132] Además, estudios en líneas celulares cancerígenas han demostrado que Gremlin produce migración celular, invasión y proliferación por mecanismos independientes de BMP y de VEGFR2, lo que sugiere que otros receptores están involucrados en la respuesta oncogénica mediada por Gremlin.[96]

En esta tesis hemos observado que la activación de la ruta del VEGFR2 causada por Gremlin se produce por un mecanismo independiente de BMPs, como lo demostramos en los estudios *in vitro* en presencia de distintas BMPs tales como BMP-2, BMP-4 y BMP-7, en donde no se ven modificados los niveles de fosforilación de VEGFR2.

El rol de Gremlin en la respuesta inflamatoria ha sido muy poco estudiado. Un estudio *in vitro* ha descrito a Gremlin como un inhibidor de la quimiotaxis de monocitos.[31] Un estudio posterior en monocitos/macrófagos ha demostrado que Gremlin se une al factor inhibitorio de la migración de macrófagos (MIF) y actúa como un antagonista endógeno de MIF.[156] La relación de Gremlin/monocitos *in vivo* es controvertida. En un modelo de ateroesclerosis experimental en ratones *knockou*t para ApoE, la administración de Gremlin redujo el contenido de macrófagos en las placas ateroescleróticas y atenuó la progresión de la lesión.[156] Sin embargo, en otro estudio utilizando este modelo y siRNA contra BMPs, Gremlin y el receptor II de BMP, ha demostrado que en condiciones pro-aterogénicas, la señalización de BMP predomina, lo que favorece el reclutamiento de monocitos y la inflamación.[223] Recientemente, se ha observado que en células endoteliales en cultivo Gremlin estimula la expresión de varias quimioquinas y moléculas de adhesión celular.[35] En esta tesis hemos demostrado *in vivo* que Gremlin induce una respuesta inflamatoria en el riñón, caracterizada por inducción de genes pro-inflamatorios

(quimioquinas, moléculas de adhesión y citoquinas) e infiltración de monocitos/macrófagos y linfocitos a las 48 horas. El bloqueo de la señalización de VEGFR2, mediante el tratamiento con el inhibidor de la quinasa de VEGFR2, SU5416, inhibe la inflamación renal inducida por Gremlin.

Los efectos de Gremlin vía VEGFR2, sobre genes pro-inflamatorios han sido confirmados en células tubulares en cultivo, mediante el inhibidor farmacológico SU5416 o silenciamiento génico de VEGFR2. Nuestros datos demuestran que el receptor VEGFR2 está involucrado en la respuesta inflamatoria renal activada por Gremlin.

3. Gremlin activa NF-κB como principal mecanismo implicado en la regulación de la respuesta inflamatoria renal

Varios autores han investigado los mecanismos intracelulares activados en respuesta a Gremlin. La mayoría de los estudios han sido realizados en células endoteliales *in vitro*, y han demostrado un incremento en la fosforilación de proteínas, producción de ROS, producción de adenosina monofosfato cíclica y activación de factores de transcripción, incluyendo la proteína de unión al elemento de respuesta de la adenosina monofosfato cíclica (CRE) y el NF-κB.[35,149]

Entre los mecanismos de señalización intracelular involucrados en la regulación de las respuestas inmunes inflamatorias, el factor NF-κB tiene especial relevancia.[212] En enfermedades renales humanas, como por ejemplo en nefropatía diabética, la actividad de la vía canónica del NF-κB evaluada por estudios histológicos, se correlaciona con un aumento de parámetros pro-inflamatorios.[145,215,282]

En nuestros estudios hemos demostrado que una de las respuestas tempranas de la activación de Gremlin/VEGFR2 *in vivo* es la activación de la vía canónica de NF-κB, observada a los 5 minutos después de la administración de Gremlin. La activación de la vía canónica de NF-κB en respuesta a Gremlin, implica la fosforilación de Iκ-Bα, lo que conlleva a su degradación por proteólisis, la fosforilación de la subunidad NF-κB p65 y la translocación nuclear del complejo NF-κB activo, donde actúa como un factor de transcripción.[212] Los análisis *in vitro* realizados, utilizando el inhibidor de la quinasa de VEGFR2, SU5416, o por silenciamiento génico de VEGFR2, demuestran que Gremlin a través de VEGFR2 activa la vía de NF-κB y regula la activación de varios genes pro-inflamatorios que se encuentran bajo el control de NF-κB.

Muchos modelos experimentales han demostrado que el bloqueo de NF-κB mediante varias aproximaciones, como por ejemplo sobre-expresión de IκB, oligonucleótidos *decoy* para NF-κB, inhibidores farmacológicos de NF-κB, inhibidores de ECA, estatinas y antioxidantes, atenúa la inflamación renal y mejora la progresión de la enfermedad.[43,44,125,152,196,199] En esta tesis hemos observado que la inflamación renal inducida por Gremlin *in vivo*, fue inhibida cuando la vía de NF-κB fue bloqueada utilizando el inhibidor farmacológico de NF-κB, Parthenolide. Estos resultados sugieren que Gremlin tras unirse al receptor VEGFR2 en células túbulo-epiteliales, activa la vía señalización de NF-kB produciendo una respuesta inflamatoria local y el reclutamiento de células inflamatorias en el riñón (**Figura 10**).

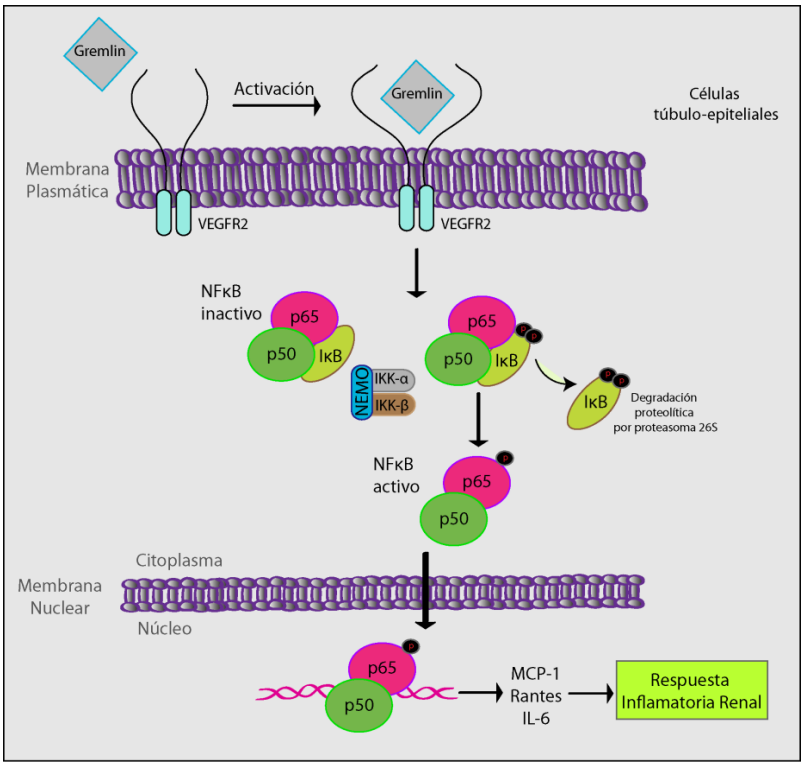

Figura 10: *Gremlin activa la vía de NF-kB en riñón.*

4. El daño renal experimental se asocia a la activación de Gremlin/VEGFR2

A pesar del gran número de investigaciones realizadas para determinar el rol de VEGF en la progresión de la enfermedad renal, esto no ha sido claramente demostrado. VEGF mediante la inducción de neo-angiogénesis y la modulación de eventos asociados a la hipoxia, puede

ejercer efectos beneficiosos o perjudiciales, dependiendo de la patología y la etapa del daño. Así, en ciertos contextos si la neo-angiogénesis es defectuosa puede exagerar la injuria.[132]

Estudios previos en el modelo de UUO han descrito la activación temprana de VEGFA a las 24 horas. Sin embargo, a tiempos más largos la expresión de VEGFA disminuyó.[117,229] Consecuentemente, los niveles reducidos de VEGFA en el UUO, podría ser el resultado de la expresión de múltiples factores además de la hipoxia y de la expresión de HIF-1.[235] En esta tesis hemos descrito que en el modelo de UUO de 5 días, los niveles génicos de VEGFA no se encuentran elevados, por el contrario si se observa una sobre-expresión renal de Gremlin. Estudios en modelos animales de nefropatía diabética, la inhibición de VEGFA ha generado resultados opuestos, algunos estudios demuestran protección pero en otros no se observan efectos beneficiosos,[101,216,217,230] desafortunadamente en estos estudios la expresión de Gremlin no fue evaluada.

En esta tesis hemos descrito que en los modelos de daño renal experimental, por UUO y por administración sistémica de Ang II, y en pacientes con distintas nefropatías, la sobre-expresión de Gremlin está asociada a la activación de la vía de VEGFR2 en el riñón, apoyando la hipótesis de que Gremlin vía VEGFR2 puede contribuir al daño renal.

En el modelo de UUO la sobre-expresión de la forma soluble del receptor VEGFR2 atenúa la fibrosis durante el daño renal.[117] En nuestros estudios hemos observado que la inhibición de la quinasa del receptor VEGFR2 disminuye la expresión renal de Gremlin, disminuyendo la expresión de marcadores inflamatorios, el número de células infiltrantes y mejora el daño renal en el modelo experimental de UUO. Estos datos sugieren que el bloqueo de Gremlin/VEGFR2 podría ser una interesante diana terapéutica para las enfermedades renales.

Varios autores sugieren que Gremlin podría ser considerado un mediador de daño en nefropatía diabética. Estudios experimentales en un modelo de diabetes inducida por estreptozotocina utilizando ratones *knockout* heterocigotos para grem1 o silenciamiento génico de Gremlin han demostrado atenuar lesiones renales.[193,280] En biopsias humanas de nefropatía diabética se ha demostrado sobre-expresión de Gremlin, principalmente en pacientes con daño renal severo y fibrosis túbulo-intersticial.[46] En este estudio hemos observado co-localización de Gremlin y activación de VEGFR2 en pacientes con diferentes nefropatías, aunque las

consecuencias funcionales faltan por ser determinadas. Estudios previos han descrito sobre-expresión de VEGFA en biopsias renales y plasma de pacientes con diabetes tipo I y tipo II,[76,78] lo que conduce a la hipótesis de que el aumento de VEGFA en diabetes es perjudicial para la función glomerular. En ratones transgénicos, la sobre-expresión de VEGFA en podocitos se asocia con algunas características de nefropatía diabética tales como el engrosamiento de la membrana basal glomerular y la proteinuria.[224,240,241] Las acciones autocrinas de VEGF en podocitos se extienden más allá de la isoforma VEGFA, involucrando los receptores VEGFRs. El receptor soluble VEGFR1 regula la morfología del podocito por su unión a microdominios lipídicos, y contribuye a preservar la homeostasis renal [2]. Sin embargo, se ha descrito que en podocitos no se expresan niveles detectables de transcrito o proteína de VEGFR2 *in vivo.*[224] Además, ratas inyectadas con el receptor soluble VEGFR1 desarrollan hipertensión, endoteliosis y proteinura, similar a aquellas lesiones vistas en ratones VEGF haploinsuficientes específico de podocitos.[52] Es importante destacar que en nefropatías progresivas humanas hemos encontrado que la sobre-expresión de Gremlin y la activación de VEGFR2 se encuentran en las células túbulo-intersticiales, mostrando claramente una distribución espacial diferente de VEFGA y patrón de expresión de Gremlin/VEGFR2.

5. Gremlin contribuye a la fibrosis renal

Diversos estudios muestran la participación de Gremlin en patologías fibróticas, incluyendo fibrosis hepática y enfermedades pulmonares (particularmente hipertensión pulmonar y fibrosis pulmonar idiopática) y la fibrosis miocárdica.[15,26,36,156,161,162] La fibrosis crónica representa la vía final común en enfermedades renales progresivas. En modelos de diabetes experimental, el bloqueo de Gremlin retarda la progresión de la enfermedad y disminuye la fibrosis renal. Así en ratones heterocigotos *knockout* para grem1 se atenúan proteínas relacionadas con MEC como Fibronectina y CTGF,[193] y el bloqueo de Gremlin endógeno mediante silenciamiento génico inhibe la proliferación celular, la apoptosis, la acumulación de Colágeno tipo IV en el glomérulo y atenúa la proteinuria.[280] Además, utilizando un ratón transgénico que sobre-expresa Gremlin de forma específica en la célula tubular, hemos observado que existe una mayor susceptibilidad al daño renal agudo causado por la administración de ácido fólico.[48]

5.1. Gremlin induce producción de mediadores pro-fibróticos y proteínas de matriz extracelular en fibroblastos renales en cultivo

En riñones normales existen pocos fibroblastos residentes, pero en condiciones patológicas el número de fibroblastos aumenta, se activan y contribuyen a la síntesis de MEC, principalmente Colágeno tipo I en el área túbulo-intersticial, así inducen fibrosis y pérdida de función renal. En distintos tipos celulares se ha descrito el efecto fibrogénico directo de Gremlin. En astrocitos de la cabeza del nervio óptico y en células de la lámina cribosa, Gremlin regula proteínas relacionadas con MEC.[287] En células mesangiales Gremlin incrementa la proliferación celular y la acumulación de MEC a través de la vía ERK.[81] En fibroblastos dérmicos, IL-6 media la síntesis de Colágeno dependiente de la producción de Gremlin y de la activación de la señalización de TGF-β/Smad.[172]

Nuestros datos *in vitro* demuestran que en fibroblastos renales, la estimulación con Gremlin aumenta la expresión de factores pro-fibróticos, incluyendo TGF-β1, CTGF y PAI-1 y la producción de Colágeno tipo I y Fibronectina, tanto liberados al medio extracelular como asociado a células. Estos resultados sugieren que Gremlin al activar la síntesis de proteínas de MEC por fibroblastos renales, podría contribuir a la excesiva acumulación de matriz en el área túbulo-intersticial, una característica clave de la fibrosis renal (**Figura 11**).

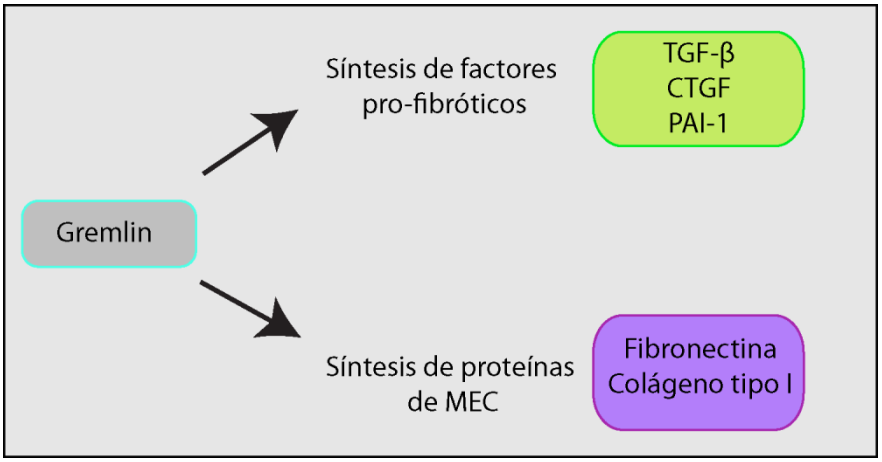

Figura 11: *Efecto fibrogénico de Gremlin en fibroblastos murinos.*

5.2. Gremlin induce transición epitelio-mesenquimal en células túbulo-epiteliales en cultivo

El evento celular clave en la fibrosis es el fibroblasto activado.[164] Una importante fuente de miofibroblastos es el epitelio renal dañado, generados a partir del proceso de TEM.[275] Gremlin también podría contribuir a la fibrosis por inducir TEM en células epiteliales tubulares. Células HK2 tratadas con Gremlin pierden sus características epiteliales, incluyendo la desaparición de proteínas epiteliales como E-cadherina y pan-Citoqueratina, y cambio a fenotipo tipo miofibroblasto, caracterizado por inducción de marcadores mesénquimales y de miofibroblasto como α-SMA, Vimentina y FSP-1 y activación de MMP-9, una enzima clave involucrada en la degradación de la membrana basal epitelial. Gremlin también induce TEM en las células epiteliales de la vía respiratoria[133] y en las células cancerígenas.[232]

Aunque la contribución de la TEM a la fibrosis renal es objeto de un intenso debate,[111,273] la pérdida de las propiedades epiteliales de las células epiteliales tubulares, incluyendo permeabilidad y la polaridad, puede resultar en la disminución de la viabilidad y contribuir a la lesión renal.[111,182] Todo esto demuestra que la inducción de TEM es el paso inicial en el daño renal y una importante diana terapéutica (**Figura 12**).

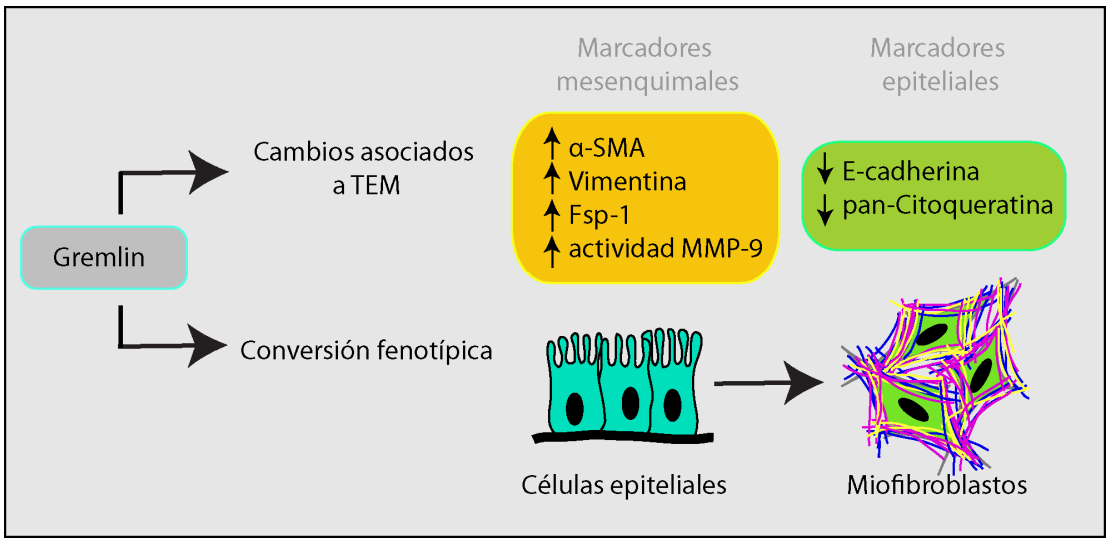

Figura 12: *Proceso de TEM inducido por Gremlin en células túbulo-epiteliales.*

5.3. Gremlin es un mediador de las acciones pro-fibróticas de TGF-β en células renales en cultivo.

En respuesta a un daño, las células tubulares renales incrementan la síntesis de TGF-β1, que es considerada la principal citoquina pro-fibrogénica del riñón, siendo un mediador clave de fibrosis renal.[20,277] En células renales en cultivo, TGF-β1 induce la expresión de Gremlin, incluyendo células mesangiales, células túbulo-epiteliales,[138,147] y como hemos descrito en esta tesis, en fibroblastos.

Varios autores sugieren una potencial relación entre las respuestas de TGF-β1 y Gremlin. En enfermedades renales progresivas se ha descrito que la expresión de Gremlin se asocia con sobre-expresión de TGF-β1 principalmente en zonas de fibrosis túbulo-intersticial.[28,46,147,160,193] Nuestro estudio *in vitro* demuestra que el bloqueo de Gremlin endógeno, por un siRNA específico, inhibe la sobre-expresión de genes pro-fibróticos y la producción de MEC inducidos por TGF-β1 en fibroblastos renales.

TGF-β1 es el mayor promotor de la TEM durante la embriogénesis, progresión del cáncer y fibrosis.[155] De hecho, la adición de TGF-β1 a células epiteliales en cultivo es una manera muy eficaz de inducir TEM en diferentes epitelios. El silenciamiento génico de Gremlin inhibe cambios en TEM mediados por TGF-β1 en células túbulo-epiteliales. Nuestros datos sugieren que Gremlin podría actuar como un mediador pro-fibrótico de las acciones de TGF-β1 (**Figura 13**).

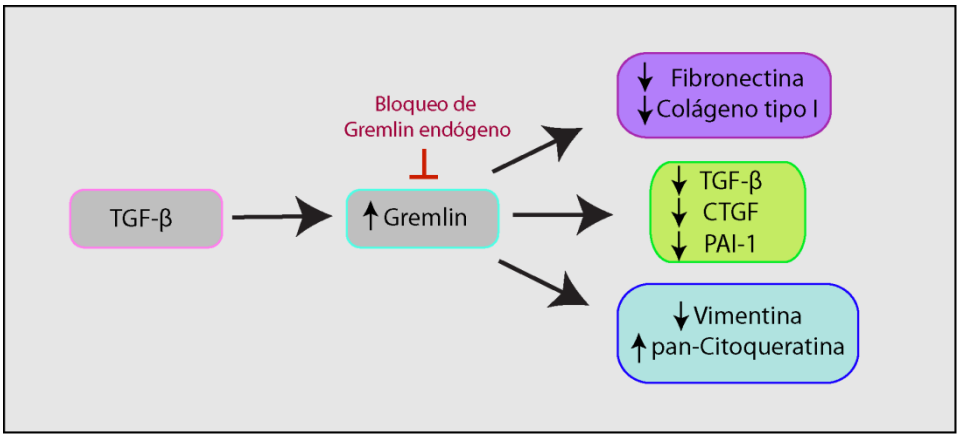

Figura 13: *Acciones pro-fibróticas de TGF-β mediadas por Gremlin.*

Gremlin heterodimeriza con BMP-2, BMP-4 y BMP-7, previniendo su interacción con receptores específicos y se cree que esta capacidad es la responsable del rol crítico de Gremlin durante el proceso de nefrogénesis, fibrosis y cáncer.[148] En pulmones de ratones expuestos a asbestos y en fibrosis pulmonar, la sobre-regulación de Gremlin fue asociada con la inhibición de la señalización BMP, demostrado por la reducción de los niveles de Smad 1/5/8.[26,161] Un mecanismo similar ha sido sugerido en TEM inducida por ácido aristolóquico.[114] BMP-7 y BMP-2 han demostrado atenuar la fibrosis intersticial renal inducida por TGF-β1 mediante la reversión del proceso de TEM.[259,268] En un reciente estudio hemos observado que Gremlin activa la vía de las Smad por una vía independiente de las BMPs, aunque el receptor implicado no ha sido evaluado.[191] Sin embargo, mecanismos independientes de BMPs podrían mediar las acciones intracelulares de Gremlin.

5.4. Gremlin regula eventos pro-fibróticos vía VEGFR2

En esta tesis hemos observado que el receptor VEGFR2 está implicado en las respuestas pro-fibróticas de Gremlin. En los estudios *in vitro*, el inhibidor SU5416 revierte los cambios asociados a TEM producidos por Gremlin. Además, el bloqueo de VEGFR2 inhibe la expresión de genes pro-fibróticos (TGF-β1, CTGF), proteínas relacionadas a MEC (Fibronectina) y factores asociados a TEM (Vimentina). Nuestros estudios *in vivo*, utilizando un modelo de inyección de Gremlin en parénquima renal de ratón, muestra un aumento significativo de genes pro-fibróticos en riñón, incluyendo TGF-β y PAI-1, efecto que fue revertido con el inhibidor de la quinasa del VEGFR2. Además, en un modelo de daño renal por UUO, caracterizado por inducción de Gremlin a nivel renal, el bloqueo de VEGFR2 también disminuye la sobre-expresión de factores pro-fibróticos y de proteínas relacionadas a MEC, así como la acumulación de Fibronectina en el riñón.

Nuestro estudio demuestra por primera vez el efecto directo de Gremlin en la regulación de eventos fibróticos en células túbulo-intersticiales renales en cultivo. Además Gremlin es un mediador de TGF-β1, un factor clave de fibrosis renal. En conjunto estos datos indican que Gremlin, vía VEGFR2 regula fibrosis renal *in vitro* e *in vivo*.

El arsenal terapéutico actual para el tratamiento de enfermedades renales progresivas es limitado. Resultados negativos con bloqueadores de TGF-β1 remarcan la importancia de encontrar nuevas dianas terapéuticas. Nuestros resultados indican que Gremlin podría ser un

importante modulador de fibrosis renal y abre futuras oportunidades para abordar Gremlin como una nueva terapia anti-fibrótica para enfermedades renales crónicas.

6. Gremlin activa la vía de señalización Notch mediante VEGFR2

Gremlin y la vía de señalización Notch participan en desarrollo embrionario, en donde ambos tienen un importante rol durante la nefrogénesis.[5,73,95,134,137,148] Sin embargo, existen pocos trabajos que relacionen Gremlin con la vía de señalización Notch en condiciones patológicas.[245] Como sucede con muchos genes que participan en desarrollo, estos se encuentran inactivos en riñón normal adulto y se reactivan durante el daño en tejido adulto.[13,46,105,167,244] Nuestros estudios *in vivo* e *in vitro* demuestran que Gremlin regula la expresión y síntesis de componentes de la vía Notch en el riñón. Además, Gremlin induce la translocación nuclear de Notch-1 activo y la sobre-regulación de la expresión génica de Hes-1, el principal efector de Notch, demostrando claramente la activación de la ruta. En células túbulo-epiteliales en cultivo, hemos demostrado que el bloqueo farmacológico de VEGFR2, utilizando el inhibidor de la quinasa de VEGFR2, SU5416, o por silenciamiento de génico, inhibió la activación de Notch inducida por Gremlin. Por otra parte, *in vivo*, la activación de la vía de señalización Notch inducida por Gremlin fue bloqueada al tratar los ratones con el inhibidor de la quinasa de VEGFR2. Estos datos muestran claramente que VEGFR2 es el receptor implicado en la activación de Notch causada por Gremlin en riñón.

La participación de la vía Notch en progresión de daño renal es un tema controvertido. En algunos modelos experimentales de daño renal, incluidos modelos de fracaso renal agudo, como administración de ácido fólico en ratones, el bloqueo de la vía Notch mejora el daño renal, principalmente por inhibir la proliferación de fibroblastos, y por tanto, disminuye la fibrosis. Sin embargo, el efecto directo de Notch en la regulación de eventos pro-fibróticos no ha sido claramente demostrado. En un estudio previo, en el modelo de UUO, la administración del inhibidor de la γ-secretasa, DAPT disminuyó la expresión génica de Fibronectina y Colágeno tipo I.[13] En esta tesis hemos ampliado este estudio, mostrando que DAPT disminuye la expresión de genes pro-fibróticos incluyendo TGF-β y PAI-1, y acumulación renal de Fibronectina. Además, en el modelo de administración de Gremlin, DAPT disminuyó la expresión de genes pro-fibróticos. Asimismo, en células túbulo-epiteliales en cultivo, DAPT

revierte los cambios asociados a TEM producidos por Gremlin, así como también disminuye proteínas de MEC y factores pro-fibróticos, indicando que Gremlin regula la TEM a través de la vía de señalización Notch. Todos estos datos sugieren que el bloqueo de la vía Notch podría representar una nueva opción terapéutica para frenar la fibrosis renal (**Figura 14**).

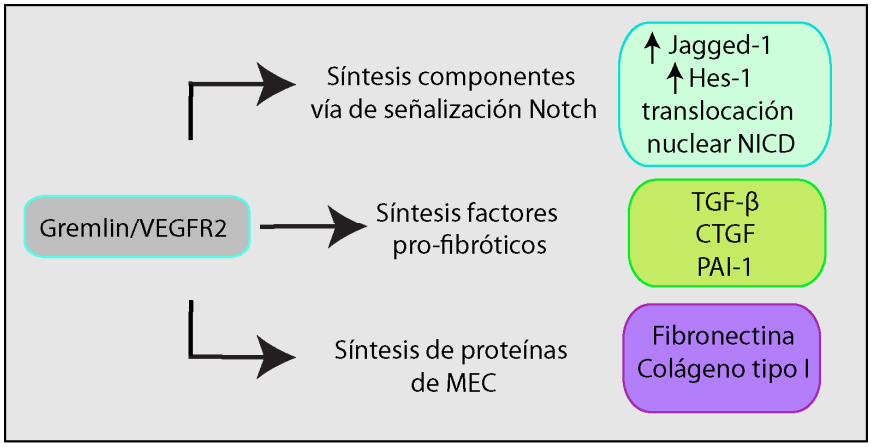

Figura 14: *Acciones de Gremlin/VEGFR2*

7. Angiotensina II no activa la vía Notch a nivel renal

La Ang II participa de forma activa en la progresión del daño renal, al ser capaz de regular diversos procesos patológicos, como inflamación y fibrosis. Los fármacos que bloquean las acciones de Ang II, como los inhibidores de la enzima convertidora de la angiotensina (IECAs) y los antagonistas de los receptores AT1 de la Ang II (ARAII), son una de las mejores estrategias terapéuticas para el tratamiento de las enfermedades renales progresivas, debido a sus acciones más allá del control de la presión sanguínea, presentando efectos órgano protectores.[41,199]

En esta tesis hemos observado que la Ang II *in vivo*, utilizando un modelo experimental de administración sistémica de este péptido en ratas, a una dosis que causa daño túbulo-intersticial, así como en un modelo de ratas espontáneamente hipertensas que presentan albuminuria y fibrosis túbulo-intersticial, no se activa la vía Notch en el riñón, en concreto no se regula la síntesis de Jagged-1 y no se observa activación de Notch-1.

En células renales en cultivo, incluidos podocitos, células túbulo-epiteliales y fibroblastos, hemos descrito que la ruta Notch no está activada en respuesta a Ang II. Nuestros

estudios *in vitro* claramente demuestran que aunque TGF-β activa la vía Notch en células renales,[153,169,263] Ang II no regula este sistema en el riñón, demostrando una diferencia clave entre los mecanismos activados por ambos factores. En el modelo experimental UUO, el tratamiento con el antagonista del receptor AT1 mejora la inflamación y la fibrosis renal, por inhibición local de quimioquinas y factores profibróticos, incluyendo TGF-β como ha descrito previamente nuestro grupo,[53] pero hemos observado que no disminuye la expresión renal de Jagged-1, como se ha descrito en esta tesis. Estos datos claramente indican que el sistema de señalización Notch/Jagged no está involucrado en daño renal asociado a Ang II e hipertensión, al menos en los modelos experimentales renales evaluados y a los tiempos estudiados.

En un amplio rango de enfermedades renales se ha descrito la activación de la vía Notch.[158] En biopsias de pacientes con nefropatía membranosa progresiva, hemos observado que la activación de Jagged-1 y Hes-1 se correlaciona con el grado de fibrosis túbulo-intersticial y con el aumento de TGF-β, confirmando el amplio estudio previo que ha demostrado activación de Jagged-1, Jagged-2 y Notch-1 en nefropatías progresivas humanas,[158] así como los datos previos de nuestro grupo en nefropatía diabética.[245]

Por otro lado, en biopsias de pacientes con nefropatía hipertensiva, con diferentes grados de fibrosis túbulo-intersticial, hemos demostrado que la vía Notch no está activada en el riñón, ampliando y confirmando nuestros estudios previos *in vitro* y en modelos animales de hipertensión y de daño mediado por Ang II, trasladando a la patología humana la hipótesis de que Ang II no regula la ruta Notch en el riñón adulto.

La relación entre Ang II y TGF-β en fibrosis ha sido bastante estudiada.[28,41,200,201,253] Se ha demostrado que TGF-β1 actúa como un mediador de la fibrosis renal inducida por Ang II y que ambos factores comparten varios mecanismos intracelulares involucrados en la regulación de la acumulación de MEC.[200,253] En células túbulo-epiteliales, hemos observado que aunque Ang II aumenta los niveles de TGF-β, esta producción de TGF-β endógeno no es suficiente para activar la vía Notch. Esta afirmación confirma nuestros hallazgos *in vivo* en los modelos de daño renal inducido por Ang II e hipertensión, ambos caracterizados por sobre-expresión de TGF-β1 y fibrosis y ausencia de la activación de la vía Notch, así como los datos en el modelo de UUO, que en respuesta al antagonista AT1 no regula Jagged-1, pero disminuye TGF-β y el daño renal.

Muchos trabajos han demostrado que la vía Notch/Jagged es esencial para la función epitelial y contribuye a la TEM en embriogénesis y cáncer.[64,154,169] En células epiteliales tubulares en cultivo, la inhibición de la vía Notch por bloqueo farmacológico de la γ-secretasa, inhibe cambios fenotípicos asociados a TEM inducidos por TGF-β1[169,264] y como hemos descrito en esta tesis, por Gremlin. En estas células Ang II y TGF-β inducen TEM por mecanismos comunes, incluyendo la vía SMAD y la cascada de las MAPK.[28,189] Cabe destacar que los datos presentados en esta tesis, demuestran que la inhibición de la γ-secretasa no modula la TEM inducida por Ang II, señalando un mecanismo de acción diferente entre Ang II, TGF-β y Gremlin. Sin embargo, hay que remarcar que la contribución de la TEM a la fibrogénesis renal es un tema de intenso debate.[64,100,122,272,275] En este sentido, en un modelo transgénico de ratón, la activación de Notch específica en células tubulares e intersticiales induce daño renal, caracterizado por un incremento de proliferación celular en ambos tipos celulares y fibrosis, pero no fueron detectados cambios en marcadores de TEM.[13] Además, hemos observado, en células tubulares en cultivo que el ligando de Notch, Jagged-1 induce la transición a un fenotipo tipo fibroblasto y cambios en marcadores de TEM, tales como pérdida de la proteína epitelial pan-Citoqueratina e inducción del marcador mesenquimático Vimentina, lo que demuestra la importancia de la activación de la vía Notch/Jagged-1 en la regulación de la TEM, por lo menos *in vitro.*

Estudios previos han demostrado que el bloqueo de Notch no inhibe la expresión de algunos genes profibróticos tales como CTGF, trombospondina y MMP-9 inducidos por TGF-β[169] y como hemos observado en esta tesis PAI-1. CTGF ha sido descrito como un mediador profibrótico clave de Ang II y TGF-β en varios tipos celulares, incluyendo células renales.[202,203,278] PAI-1 participa en fibrosis vascular inducida por Ang II, independiente de TGF-β.[190,248] Hemos demostrado que estos genes (CTGF, PAI-1, MMP-9, Vimentina y Fibronectina) son activados por Ang II independientemente de la activación de Notch. Estos datos indican que varios de los eventos pro-fibróticos inducidos por TGF-β y Ang II son independientes de la activación de la vía Notch. Diversos estudios experimentales han observado que el uso de inhibidores farmacológicos o ligandos solubles de Notch mitigan el fallo renal.[99,167] En esta tesis hemos observado que DAPT disminuye la fibrosis renal asociada a UUO. Además, estudios en

ratones transgénicos con deleción de Notch a nivel de podocitos han demostrado la implicación de esta vía en fibrosis túbulo-intersticial.[13]

La activación de Notch específica de podocito daña severamente la barrera de filtración glomerular en el riñón. La inhibición de la vía Notch en podocitos mediante ablación genética específica del coactivador RBP-Jκ de Notch o por bloqueo farmacológico de la γ-secretasa, revierte el daño glomerular y re-establece la barrera de filtración.[99] La sobre-expresión de TGF-β produce daño podocitario, proteinuria y glomérulo-esclerosis progresiva.[214] Además, la inhibición de Notch modula la apoptosis de podocitos dependiente de p-53 y mediada por TGF-β.[167] En cultivo de podocitos humanos, TGF-β1, VEGF y alta glucosa activan la vía Notch e inducen apoptosis de podocitos.[116,167]. Sin embargo, en estas células Ang II no induce apoptosis[210] y como hemos descrito aquí no incrementa la producción de Jagged-1. En un modelo de nefropatía diabética en rata, la inhibición farmacológica de la vía Notch mejora la proteinuria,[116] lo que demuestra que la inhibición específica de Notch en podocito podría ser una buena opción terapeútica para enfermedades proteinúricas, caracterizada por pérdida de podocitos por apoptosis.

Se ha descrito divergente funcionalidad de Notch dependiendo del tipo celular. En la vasculatura, Notch-3 regula el tono vascular y el crecimiento celular/apoptosis.[21,27] Además, en estas células Ang II inhibe la expresión de Notch-3,[27] mientras que en células túbulo-epiteliales, ni TGF-β ni Ang II modulan la expresión génica de Notch-3. En el riñón, la sobre-regulación de Notch-3 fue solo observada en progenitores renales en glomérulo-esclerosis humana,[108] lo que apoya el rol de Notch en regeneración renal.

Comprender la fina regulación del sistema Notch en el riñón dañado es importante, ya que la señalización Notch podría participar tanto en la regeneración como en la progresión del daño renal. En riñones adultos existe una población de células residentes con actividad progenitora que expresan miembros de la familia Notch.[29] En daño renal inducido por ácido fólico, la inhibición de Notch no modifica el daño renal agudo y los niveles de creatinina (un marcador de recuperación renal), pero mejora las lesiones renales y la fibrosis, observado a los 7 días.[13] Cabe destacar que la activación de Notch fue detectada sólo en células proliferativas. En un modelo de necrosis tubular aguda, inducido por isquemia-reperfusión, el tratamiento con

el ligando de Notch *Delta-like 4,* facilita la recuperación renal por incremento de la proliferación celular.[67]

Estos datos sugieren que los efectos beneficiosos descritos de la inhibición de Notch podrían ser debido a la modulación de la proliferación celular. Además, la activación de Notch en progenitores renales humanos estimula la proliferación celular, mientras que su inhibición es requerida para la diferenciación hacia el linaje de podocito. De hecho, la activación persistente de Notch induce muerte de podocitos por catástrofe mitótica.[108] En modelos murinos de glomérulo-esclerosis focal y segmentaria, la inhibición de Notch reduce la pérdida de podocitos y mejora la proteinuria durante fases iniciales de daño glomerular, pero la inhibición de Notch en fases regenerativas de daño glomerular reduce la proliferación de progenitores celulares y empeora la proteinuria y la glomérulo-esclerosis.[108]

Existe una falta de tratamiento eficaz para enfermedades renales crónicas. El efecto beneficioso de la inhibición de Notch en enfermedad glomerular proteinúrica experimental, incluyendo nefropatía diabética, muestra la importancia de la activación de Notch en fallo podocitario. Sin embargo, hemos descrito que la vía Notch no estaría involucrada en eventos fibróticos inducidos por Ang II. Este péptido contribuye a la progresión del daño renal, induciendo eventos relacionados con fibrosis y su bloqueo retrasa la progresión de la enfermedad renal en humanos. Aunque hay algunos ensayos clínicos que utilizan inhibidores de la γ-secretasa para diversas enfermedades como el Alzheimer y la leucemia,[99] nuestros estudios experimentales no apoyan el potencial efecto beneficioso de estos fármacos en enfermedades renales mediadas por Ang II. Por el contrario, nuestros estudios confirman que en patologías progresivas humanas la activación de la vía Notch se asocia a fibrosis renal. En conjunto, nuestros resultados muestran la compleja regulación de la vía Notch en el riñón e indican que son necesarios más estudios para mejorar las actuales aproximaciones terapéuticas para limitar la progresión del daño renal, antes del uso de inhibidores de la γ-secretasa para enfermedades humanas.

V. CONCLUSIONES

1. VEGFR2 es un receptor funcional de Gremlin en riñón.

2. Gremlin *in vivo* da lugar a una respuesta inflamatoria renal mediada por la rápida activación de la ruta de NF-κB y la regulación de genes bajo su control y el reclutamiento de células inflamatorias vía receptor VEGFR2.

3. En modelos experimentales de daño renal y en pacientes con diversas nefropatías, la inducción de Gremlin y la activación de la vía VEGFR2 sugieren un nuevo mecanismo en el daño renal.

4. En estudios *in vitro* hemos demostrado que Gremlin es un factor pro-fibrótico, que regula la síntesis de matriz extracelular y causa transición epitelio-mesequimal y actúa como es un mediador de las acciones pro-fibróticas de TGF-β.

5. Gremlin, via VEGFR2, activa la vía de señalización Notch en riñón *in vivo* e *in vitro* y esta activación está asociada a la regulación de procesos fibróticos.

6. La Ang II no activa la ruta Notch, mostrando una clara diferencia con otros factores pro-fibróticos, como TGF-β y Gremlin.

7. En biopsias renales humanas de pacientes con nefropatías progresivas se observó activación de la vía Notch, pero no en pacientes con nefropatía hipertensiva, ni en modelos experimentales de hipertensión.

   En conjunto, estos datos sugieren que Gremlin podría ser considerado como un nuevo mediador de daño renal a través de la activación del VEGFR2, y muestran que el eje Gremlin/VEGFR2 podría contribuir a la progresión del daño y ser una nueva diana terapéutica en las patologías renales.

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VII. ANEXO

Los resultados presentados en esta tesis han sido publicados en:

- Angiotensin II Contributes to Renal Fibrosis Independently of Notch Pathway Activation. **Carolina Lavoz,** Raquel Rodrigues-Diez, Alberto Benito-Martin, Sandra Rayego-Mateos, Raúl R. Rodrigues-Diez, Matilde Alique, Alberto Ortiz, Sergio Mezzano, Jesús Egido, Marta Ruiz-Ortega. PLoS One. 2012;7(7):e40490.

- Gremlin Is a Downstream Profibrotic Mediator of Transforming Growth Factor-Beta in Cultured Renal Cells. Rodrigues-Diez R, **Lavoz C,** Carvajal G, Rayego-Mateos S, Rodrigues Diez RR, Ortiz A, Egido J, Mezzano S, Ruiz-Ortega M. Nephron Exp Nephrol. 2013;122(1-2):62-74.

- Translational study of the Notch pathway in hypertensive nephropathy. **Lavoz C**, Droguett A, Burgos ME, Carpio DJ, Ortiz A, Egido J, Mezzano S, Ruiz-Ortega M. Nefrologia 2014;34(3):369-76.

Otros trabajos publicados durante el desarrollo de la tesis:

- Connective tissue growth factor (CTGF): a key factor in the onset and progression of kidney damage. Sánchez-López E, Rodrigues Díez R, Rodríguez Vita J, Rayego Mateos S, Rodrigues Díez RR, Rodríguez García E, **Lavoz C**, Selgas R, Mezzano S, Egido J, Ortiz A, Ruiz-Ortega M. Nefrologia. 2009; 29(5):382-91.

- Statins inhibit angiotensin II/Smad pathway and related vascular fibrosis, by a TGF-β-independent process. Raúl Rodrigues Díez, Raquel Rodrigues-Díez, **Carolina Lavoz**, Sandra Rayego-Mateos, Esther Civantos, Juan Rodríguez-Vita, Sergio Mezzano, Alberto Ortiz, Jesús Egido, Marta Ruiz-Ortega. PLoS One. 2010;5(11):e14145.

- The C-terminal module IV of connective tissue growth factor is a novel immune modulator of the Th17 response. Rodrigues-Díez R, Rodrigues-Díez RR, Rayego-Mateos S, Suarez-Alvarez B, **Lavoz C,** Stark Aroeira L, Sánchez-López E, Orejudo M, Alique M, Lopez-Larrea C, Ortiz A, Egido J, Ruiz-Ortega M. Lab Invest. 2013; 93(7):812-24.

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- Integrin-linked kinase plays a key role in the regulation of Angiotensin II-induced renal inflammation. Alique M, Civantos E, Sanchez-Lopez E, **Lavoz C,** Rayego-Mateos S, Rodrigues-Díez R, García-Redondo AB, Egido J, Ortiz A, Rodríguez-Puyol D, Rodríguez-Puyol M, Ruiz-Ortega M. Clin Sci (Lond). 2014; 127(1):19-31.

- Gremlin activates the Smad pathway linked to epithelial mesenchymal transdifferentiation in cultured tubular epithelial cells. Raquel Rodrigues-Diez, Raúl Rodrigues-Diez, **Carolina Lavoz**, Gisselle Carvajal, Alejandra Droguett, Ana B. Garcia-Redondo, Isabel Rodriguez, Alberto Ortiz-Arduán, Jesús Egido, Sergio Mezzano, M. Ruiz-Ortega. Biomed Res Int. 2014 (Aceptado para su publicación).

<u>Los resultados de esta tesis han sido parcialmente presentados en las siguientes reuniones científicas:</u>

- Papel de la vía de señalización Notch en daño renal inducido por Hipertensión. Diferente respuesta de Angiotensina II and TGF-β. **Carolina Lavo**z, Raquel Rodrigues-Diez, Sandra Rayego, Raúl Rodrigues-Diez, Alberto Benito-Martin, Alberto Ortiz, Jesús Egido, Sergio Mezzano, Marta Ruiz-Ortega. XLI Congreso Nacional de la Sociedad Española de Nefrología, 15-18 Octubre de 2011. Sevilla, España.

- Gremlin is a mediator of high glucose and TGF-β-induced fibrosis in renal cells. Sergio A. Mezzano, Raquel Rodrigues-Díez, **Carolina Lavoz,** Raúl R Rodrigues-Díez, Sandra Rayego-Mateos, Alberto Ortiz, Jesús Egido, Marta Ruiz-Ortega. Annual Meeting ASN, 8-13 Noviembre de 2011. Philadelphia, PA, USA.

- Differential regulation by Angiotensin II and TGF-β1 of Notch Signaling in the kidney. **Carolina Lavoz,** Raquel Rodrigues-Diez, Alberto Benito-Martin, Sandra Rayego-Mateos, Raúl Rodrigues-Diez, Matilde Alique, Alberto Ortiz, Sergio Mezzano, Jesús Egido, Marta Ruiz-Ortega. 49th ERA-EDTA Congress, May 24-27, 2012. Paris.

- Gremlin via VEGFR2 Increases Profibrotic Evens in Renal Cells. Sergio A. Mezzano, Raquel Rodrigues-Diez, **Carolina Lavoz,** Alberto Ortiz, Jesus Egido, Marta Ruiz-Ortega. Nephrology, Universidad Austral, Valdivia, Chile; Cellular Biology in Renal Diseases Laboratory, Universidad Autónoma, Madrid, Spain; Nephrology, Instituto de Investigacion Sanitaria Fundación Jiménez Díaz, Madrid, Spain. Annual Meeting ASN, 30 de Octubre-4 de Noviembre de 2012. San Diego, CA, USA.

- Gremlin regula factores relacionados con fibrosis a través del receptor del factor de crecimiento endotelial vascular-2 (VEGFR2) en células túbulo-intersticiales en cultivo. **C Lavoz,** R Rodriguez-Díez, S Rayego-Mateos, R.R Rodrigues-Diez, M Orejudo, M Alique, A Ortiz, J Egido, S Mezzano, M Ruiz-Ortega. XLII Congreso Nacional de la Sociedad Española de Nefrología 6-9 de octubre de 2012, Maspalomas, Gran Canaria.

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- Angiotensina II induce la síntesis y expresión de Gremlin en el riñón. **Carolina Lavoz,** Raquel Rodrigues-Diez, Jesús Egido, Sergio Mezzano, Marta Ruiz-Ortega. 18ª Reunión Nacional de la SEH-LELHA, 6-8 de Marzo de 2013. Valencia, España.

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